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PROCEEDINGS OF THE
AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

SEVENTH ANNUAL MEETING

Cleveland, Ohio, December 30, 1912-January 1, 1913

WALDEMAR KOCH

My first duty, as President, is to pay a tribute to the memory of a worker who has fallen out of our ranks since the last meeting of our Society. Waldemar Koch was only thirty-seven years of age when he died and he was young, therefore, as the years go, but he had attained in Science a place which not rarely is the reward of later years only with others. What he did accomplish, however, was but the prologue, as it were, of a career of greater distinction which the years to come seemed abundantly to promise, and which he might hope to achieve. That hope has been denied and we who know him can but deplore our loss as well as his—ours, because he was a worthy companion enrolled with us in the scientific legion. To the older in its ranks to whom he seemed to be of the blood strain from which leaders in the legion come, as well as to the younger to whom he was a loved comrade, there remains and will remain a keen regret for his early fate, but therewith will remain also a cherished remembrance, and a thankfulness that he marched with us, even for so short a time, towards the distant watch fires of Truth.

A B MACALLUM

PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

PRESIDENTIAL ADDRESS

THE ORIGIN OF MUSCULAR ENERGY THERMODYNAMIC OR CHEMODYNAMIC?

By A. B. MACALLUM

The question of the source and the mode of production of the energy evolved in muscular contraction is one which has been regarded, ever since the experimental method was introduced in physiology, as amongst the most fundamental and the most elusive in the whole range of vital phenomena. It was early recognized that the problem required for its solution more data than were then available or were ascertainable and that the solution itself would have to wait for those data indefinitely. There were some who held that the problem was essentially an insoluble one.

There was, however, speculation and the first to advance an explanation based on rational grounds was J. R. Mayer,¹ who in 1845 propounded the view that muscle in doing work conducts itself as a thermodynamic machine or heat engine, in which heat is produced by the combustion of material in the muscle and a portion of the heat so produced is transformed into work. This view, although it had the support of a number of isolated thinkers on the subject, was not generally received and as late as 1879 Hermann² rejected it on the score that there was not a single fact to be adduced in its support. Three years later, that is, in 1882, Fick³ formulated definitely the objections to the theory. He pointed out that if muscle is a thermodynamic engine it must work in accord with the second law of thermodynamics. Now in every thermodynamic machine, as, *e g*, the steam engine, the

¹ Mayer, J. R. *Die organische Bewegung in ihren Zusammenhang mit dem Stoffwechsel*, Heilbronn, 1845

² Hermann, L. *Handbuch der Physiologie*, 1, Pt 1, p 247, 1877

³ Fick, A. *Mechanische Arbeit*, 1882

process by which work is produced can only be carried out by the passing of heat from one body of higher temperature A to another of lower temperature B under such conditions that a portion of the heat so transferred is converted into work. The quantity of heat so converted, that is, the amount of work done, can be ascertained accurately provided that the quantity of heat transferred is known as well as the temperatures of A and B and of the medium by which the transfer is accomplished and provided also the final state of this medium is the same as its initial state. The amount of work so produced is represented in the equation

$$Q_0 = Q T_0 \left(\frac{1}{T_2} - \frac{1}{T_1} \right)$$

in which Q_0 indicates the quantity of heat energy converted into work, Q the amount of heat passed from the body A of higher absolute temperature, T_1 , to the body B of lower absolute temperature, T_2 , and T_0 the absolute temperature of the medium. Under the most favorable circumstance $T_0 = T_1$ and in the most efficient engines $Q_0 = \frac{1}{4} Q$. Accordingly, in muscle, which, if a thermodynamic machine, must be considered as a highly efficient one, T_2 can only be the absolute temperature of the body or $273^\circ + 37^\circ = 310^\circ\text{C}$ and, therefore, by the equation, $T_1 = 387^\circ$ or 114°C . This involves a very much higher temperature than has been postulated to occur in muscle during contraction while the actually observed temperature after a single contraction does not exceed 0.001°C above that of the same muscle at rest. This makes it impossible to suppose that the quantity of work resulting could be developed in this manner.

That muscle can under circumstances which are of course unusual develop energy into work thermodynamically Fick does not deny but in such cases the quantity of heat transformed into work is so small that it does not help us in believing that the transformation of heat into work occurs in ordinary muscle contraction. An instance of this he gives. A muscle, extended by a weight at 20°C is warmed to 30°C . It shortens and lifts the weight. It does additional work if it is gradually unloaded at this temperature until the attached weight = 0. It is then cooled to 20°C in order to allow it to resume its ordinary or unextended

length characteristic of that temperature. It is now extended by gradually increasing weight till the latter is as great as in the first instance. It is now re-warmed to 30°C when the work done is represented by the weight raised multiplied by the distance through which it is raised. In these two cycles the muscle received heat from outside and a portion of it is transformed into work, which, however, is small in amount but its production is in accord with the second law of thermodynamics.

With the rejection of the thermodynamic hypothesis there remained two other hypotheses, one, that the energy of muscular contraction is derived from electrical energy, the other that it is derived from the energy of chemical attraction. Regarding the former little need be said. It did not attain any currency or influence speculation or stimulate experiment and, further, as a theory did not render intelligible in any respect any portion of the problem as to the origin and mode of production of the energy of muscular work.

The second of these two theories, that which postulates that muscular contraction results from the action of the attractive forces of molecules and atoms on each other had the support of Fick,⁴ Pfluger⁵ and others. This theory assumes that the molecules, on the combination of which muscular contraction depends, are arranged within the contractile substance of muscle fibre in such a way that, when the excitant action develops, they approach each other in the direction of the long axis of the muscular fibrils and thus there results a shortening of the latter. This theory is accordingly known as the chemodynamic theory. It was, until recently, practically the only resort for those who rejected the thermodynamic origin of muscular contraction but it also had its critics who claimed that it did not in the ultimate analysis afford any intelligible explanation of the phenomena of contraction. Engelmann pointed out that in a gram of muscle the elevation of temperature produced by one contraction, namely, 0.001°C or 0.001 calorie, postulated a combustion of one-four thousandth of a milligram of carbohydrate or one-four millionth of the muscle substance. This minute portion, set in a soft

⁴ Fick, A. *Medizinische Physik*, 3te Aufl., p. 206, 1885.

⁵ Pfluger, E. F. W. Ueber die physiologische Verbrennung in den lebendigen Organismen, *Arch f d ges Physiol*, x, p. 329.

watery mass, must, as required by the theory, set four million parts, less one, in motion in order to bring about contraction. There is a further inherent difficulty. The molecules which generate the movement are so sparsely distributed in the muscle substance and, therefore, so far from each other that the distance between any two of them must be far greater than the distance through which molecular attraction is effective.

Engelmann⁶ accepted the thermodynamic explanation of the origin of muscular energy. He recognized and admitted that the temperature demanded in the source of the energy in the muscle to account for the amount of work done must be high, while the temperature observed is so low that it does not exceed at the most 0.005° and usually only 0.001°C for each muscle twitch, but he explained that this is an average temperature for the whole muscle while the points where the heat is generated are infinitesimal in size. At a very minute distance from each of these points the temperature must fall so low as to be harmless to the muscle substance around each point. The muscle will no more be destroyed in this fashion than a steamship will be destroyed by heating the furnaces. "The material of combustion only will be destroyed, the vessel as a whole remains unharmed."

Engelmann advanced some facts in support of his view. He attached a moistened catgut violin string by one end, the lower, to a base and by the other to a lever which may carry a weight. Wound round the string, but at some distance from the latter, was a spiral of platinum wire whose ends were connected with the poles of a Bunsen or Grove battery of three or more cells. The base with the string and spiral was immersed in water in which at ordinary temperature the length was constant although under a weight of 25-50 grams. If a current were sent through the wire for some seconds, the water inside the spiral and about the string was heated quickly, the string immediately shortened and the lever inscribed a curve on smoked paper in many respects like the curve of the normal single twitch of a frog's muscle. In this was found what may be called a latent period, a period of quickly developing shortening followed by a period of gradually

⁶ Engelmann, Th. W. *Ueber den Ursprung der Muskelkraft*, 2te Aufl., Leipzig, 1893. On the Nature of Muscular Contraction, *Proc. Roy. Soc.*, LVII, p. 411, 1895.

decreasing relaxation. Further, the strength of the shortening power increased with the load up to a certain point. The contraction could be repeated as often as wished and the result was the same. The same phenomena were manifested by a crutchlike string.

In these experiments the heat supplied to the string was derived from the current passing through the wire. A portion of the heat received by the string was transformed into work. Engelmann associated this power to convert heat into work with the doubly-refractive material which exists in violin strings of the type he used, in crutchlike bands, as well as in muscle fibre and he regarded the doubly-refractive substance as directly concerned in this transformation.

These views of Engelmann were advanced in 1893 and again in 1895 and they were criticized by Fick¹ who maintained that they were inadequate to explain the mode of production of the energy of muscular contraction. He pointed out that the violin string shortening and relaxing in the apparatus employed by Engelmann did not fulfil the conditions demanded in a reversible thermodynamic machine of the Carnot type or of that of Clausius. Also the amount of work performed by such a string is so small as to be almost negligible. Fick calculated that the transsectional area of Engelmann's apparatus was 3 cm^2 and the length of the string 2 cm. It lifted 50 grams to a height of 0.5 mm. A muscle of like sectional area and like length would at least raise 10 kilos through the same distance, that is, it would do two hundred times as much work. Moreover Fick estimated that in a muscle of 1 cm^2 in cross section the motagmata, or doubly refracting elements which, according to Engelmann, transformed heat into work do not, all told, give a transsectional area of more than 0.01 mm^2 and yet these delicate elements are supposed to develop all the energy of muscle contraction.

Since 1895, the thermodynamic hypothesis has undergone a transformation which is largely the result of the general acceptance of the van't Hoff-Arrhenius theory of solutions. This theory postulates that substances, electrolytes and non-electrolytes, dissolved in a fluid are in a gaseous condition and thus exercise within

¹ Fick A. Einige Bemerkungen zu Engelmann's Abhandlung über den Ursprung der Muskelkraft, *Arch f d ges Physiol*, lxx, p 611, 1893

the limits of the solution gas pressures equal to their concentrations. If now two fluids, one containing solutes and the other pure, be separated by a movable, semi-permeable membrane the latter will, according to the theory, be displaced, driven as it were by the pressure of the molecules and ions of the solute, *through* the pure fluid just as the plate of a piston in a steam cylinder is driven by the head of pressure of steam behind it. Such a displacement of the membrane postulates of course that water or the pure fluid passes through the membrane, in other words, that the solution is diluted and increased in volume. If a piston rod is connected with the membrane, work, that is, mechanical energy, may be produced. It is now possible to reverse the process by partially freezing the solution and removing therefrom the ice crystals thus formed, the water from which is added to the fluid on the other side of the membrane. The latter is then displaced in the opposite direction, work is again done and the system is once more in the initial state, that is, ready to begin the cycle of operations over again.

This is indeed the Carnot principle with but a slight variation and it is the fundamental meaning of all the views based on the principle of osmotic pressure which have been advanced to explain muscular contraction. This is, therefore, the significance of the theories advanced by McDougall,⁸ Pauli⁹ and others, which account for the contraction of the sarcostyles in muscle fibres, by the passage of water into them from without, causing them to swell and thereby to shorten. This process of "Quellung" or imbibition must be due to a force inside the sarcomeres like that which operates in the solution on one side of the semi-permeable membrane and causes the water to pass through the latter from the other side, that is, the pressure, whatever may be its causation, which obtains in the interior of a sarcostyle at the moment of excitation is greater than the pressure in the sarcoplasm. The origin of this pressure may be due to a greater number of molecules, or of

⁸ McDougall, W. On the Structure of Cross-striated Muscle and a Suggestion as to the Nature of its Contraction, *Journ. of Anat. and Physiol.*, **xxxi**, p. 410, 1897, also A Theory of Muscular Contraction, *ibid.*, **xxvii**, p. 187, 1898.

⁹ Pauli, Wolfg. *Kolloidchemie der Muskelkontraktion*, Dresden and Leipzig, 1912.

molecules and ions of lactic acid, for instance, or it may be due to ion-proteins, formed from unions and proteins, which cannot pass through membranes and yet exert an osmotic pressure. This is practically Pauli's explanation of the hydratising powers of colloids on which the "Quellung" or imbibition, and thereby also the contraction of muscle fibres, are based.

It is a remarkable fact that all these theories are often classified as chemodynamic, apparently because they make use of concepts and theories of physical chemistry which, it is forgotten, are largely based on thermodynamics. It is indeed generally accepted that the ultimate source in muscle fibres of the energy of contraction is to be found ultimately in certain organic compounds which on combustion yield their energy in the kinetic form and so far it may be said that the energy of muscular contraction is chemodynamic, but the energy given out is work in a gas engine is derived from the explosive combustion of the gas employed and yet we do not consider the gas engine a chemodynamic but a thermodynamic machine.

The chemodynamic theory that was accepted by Fick, Pflüger and others was based purely on the chemical affinity between oxygen on the one hand and carbon and hydrogen of organic compounds in muscle fibres on the other, and when the muscle fibre is under the influence of excitation this affinity was supposed to be allowed free play with the result that carbon and hydrogen atoms moved rapidly toward oxygen atoms or *vice versa*. As this line of movement was supposed to be in the long axis of the muscle fibre it postulated that the latter shortened. Here the primary, the fundamental, force, the sole force concerned, was supposed to be the attraction exercised on each other by the atoms of the substances involved in the combustion, and osmotic pressure was supposed to function only to the extent of promoting the diffusion into the muscle fibres of the material undergoing combustion and the removal of the waste products.

The osmotic principle if it plays the part in promoting muscular contraction required in the theories of Pauli, McDougall and others is a thermodynamic one and it is, in consequence, open to all the objections that Fick urged against thermodynamic explanations in general of muscular contraction. There are others, one of which concerns the time which explanations based on osmo-

sis demand The process of imbibition involves the time factor It is impossible to conceive that water will flow into the sarcostyles from the sarcoplasm, not in a third or a tenth of a second, only, but as in the case of the wing muscles of insects, in less than one-two thousandth of a second Meigs tries to meet such a difficulty by suggesting that imbibition is not less rapid than it is in the case of a cotton string brought in contact with water for a fraction of a second It may be pointed out that the time involved, though less, perhaps, than a second, is much greater than that postulated in the muscle fibre and that water penetrates the interstices of a string through the action of capillarity, a factor which does not play a part in muscle contraction according to the "Quellung" theories, based, as already indicated, on osmotic pressure

Another fundamental objection to be urged against the "Quellung" theories is that the volume of each of the doubly-refracting discs of the sarcostyles in contraction is not, according to Schäfer,¹⁰ appreciably different from that of the discs in the relaxed condition and thus Hurthle¹¹ confirms McDougall, also, admits that he was unable to find an increase in volume of the sarcomeres when they contracted Schafer, further, points out that in many muscles, for example, those of insects and of vertebrates, the sarcoplasm between the sarcostyles is too small in quantity to account for the necessary increase of volume of the sarcomeres involved according to the theory—even if the whole of the sarcoplasm were absorbed by the fibrils He urges, moreover, that insect wing muscle fibres contract freely in hyperisotonic solutions, that is, fluids in which the osmotic pressure greatly exceeds that which obtains in the interior of the sarcostyles

Founded on the osmotic principle also is the theory of Zuntz,¹² advanced in 1908 This differs from the theories of McDougall and Pauli only in that it is most frankly thermodynamic Zuntz

¹⁰ Schäfer, E A On McDougall's Theory of Muscle Contraction, etc, *Quarterly Journ of Exp Physiol*, III, p 63, 1910

¹¹ Hurthle, K Ueber die Struktur der quergestreiften Muskelfasern von Hydrophilus im ruhenden und tätigen Zustand, *Arch f d ges Physiol*, CXXVI, p 125, 1909

¹² Zuntz, N Die Kraftleistung des Thierkörpers, *Festrede zur Feier des Geburtstages Sr Majestät des Kaisers, etc*, am 26 Januar 1908, Berlin, 1908

estimates that, each rod in a sarcostyle having a height of 6μ and a diameter of 1μ , there would be about 5×10^{10} of such rods in a segment of muscle of 1 cm^2 sectional area and 1 cm length and these would have a surface area of 8928 cm^2 available for osmotic work. Inside of these rods combustion takes place at the beginning of contraction and the carbon dioxide formed is sufficient under ordinary conditions to give an osmotic pressure of about 5 grams per 1 cm^2 , but as the temperature of combustion is 6400°C this pressure is raised to 162 grams per 1 cm^2 . This latter exceeds the pressure in the sarcoplasm and in consequence water quickly passes from the sarcoplasm into the sarcostyles, each rod of which swells and assumes a spherical shape and, in consequence, the muscle contracts. Immediately thereafter the heat radiates from the rods into the sarcoplasm, the pressure falls and water passes out of the rods and therewith also carbon dioxide and other products formed inside the sarcostyle at the moment of contraction. The radiation of heat, the fall of pressure and the passage of water from the rods are all coincident with and cause relaxation of the sarcostyles, that is, the muscle lengthens.

There are several objections to be urged against this theory some of which have been advanced against the "Quellung" theories. These are the enormously high temperature postulated, the rate at which diffusion of water into and from the sarcostyles must take place and the impossibility of explaining how the membrane enclosing the rods should at one moment be impermeable to carbon dioxide and in less than 0.001 second thereafter become readily permeable.

A temperature of over 6000°C in the rods of the sarcostyles is unthinkable. It would destroy the organic matter completely at the point where it would be produced and it would cause dissociation, not only of the carbon dioxide into carbon monoxide and oxygen but also of some of the water molecules into hydrogen and oxygen. Vesicles filled with carbon monoxide, hydrogen and oxygen would be formed and laceration of the internal structure of the rods would ensue.

The rate at which diffusion of water into the sarcostyles takes place is estimated by Zuntz to be extraordinarily rapid as indeed it would have to be theoretically. Assuming that oxygen diffuses

in muscle as rapidly as it does from the capillaries of the frog's lungs through the intercapillary tissue of the latter, he calculates that 0.0012 second would be required for the oxygen to pass from the sarcoplasm to the centre of a sarcofibril and that, as diffusion of a substance occurs at a rate directly as the square root of its molecular weight, water would pass from the sarcoplasm into the centre of the sarcofibrils in $\frac{0.0012 \times \sqrt{18}}{\sqrt{32}}$ second or about

0.0008 second. This would provide a velocity more than sufficiently great to account for a contraction which lasts only 0.03 second. According to the same calculation the carbon dioxide at the same tension would diffuse from the sarcofibril into the sarcoplasm in 0.0019 second but much more rapidly than this at the tension which would obtain in the sarcofibrils when the contraction develops. There would, therefore, be little or no diffusion of water into the sarcofibrils if the superficial membranes of the latter were permeable to the carbon dioxide at that moment. If impermeable how do they become permeable when relaxation is to begin? Zuntz offers no explanation of this. Indeed he is silent altogether on this point.

Zuntz estimates also that the volume of the sarcofibrils would in a strong contraction increase about 84 per cent. Here too may be urged the fact that McDougall, Schafer and Hurthle have been unable to find any increase in volume of the sarcofibrils in contraction.

It follows from all this review that the "Quellung" theories of muscle contraction present difficulties which are almost as great as the problem which they are intended to solve or explain. They are all based on the hypothesis that the breakdown of the compound responsible for the production of the heat of a muscle contraction immediately precedes or completely synchronizes with that contraction. This, as A. V. Hill¹³ has pointed out, is very doubtful. We have no means of demonstrating this synchronism if it obtains. Another assumption, sometimes explicitly, sometimes tacitly made, is that this heat is derived from the combustion of a carbohydrate—that is, of a glucose or glucose-holding

¹³ Hill, A. V. The Heat Production of Surviving Amphibian Muscles, during Rest, Activity and Rigor, *Journ. of Physiol.*, xlv, p. 466, 1912.

body, where as the work of Fletcher, Hopkins and Hill seems to indicate that it is produced by the breakdown of a lactic acid complex which sets free that acid and at the same time more heat than is produced in the complete combustion of glucose. In the presence of oxygen this complex is built up again out of the lactic acid and other products of its breakdown and in that synthesis heat energy is transformed into chemical, that is, potential energy.

These observations seem to give a new aspect to the question of the origin and mode of production of muscular energy. They do not, of course, dispose finally of the "Quellung" theories. These are, as already indicated, open to serious objections on their own score alone. What is shown is that the processes involved are not so simple as postulated in the hitherto advanced thermodynamic theories of muscular contraction.

It is evident also that we cannot account for the energy of muscular contraction on the basis of a chemodynamic process such as Verworn and others held. The difficulty involved in the acceptance of such a chemodynamic theory was clearly indicated by Engelmann and the objection is irrefutable in view of the results of Fletcher's, Hopkins' and Hill's investigations. Must we then after all fall back on some thermodynamic explanation?

My answer to this is that we have still an explanation which is not a thermodynamic one and yet is not chemodynamic in the sense of that term as employed by Fick, Pflüger and Verworn. This explanation postulates as the chief factor in muscular contraction the attraction between the molecules, constituting the superficial film of a sarcostyle and forming an interface with the sarcoplasm surrounding the sarcostyle. This attraction is the cause of the surface tension in the superficial film of every fluid, semi-fluid, or semi-viscid system and obtaining, therefore, in the superficial film of each sarcostyle. In the doubly-refractive discs of insect wing muscle fibrils the tension is not equal throughout the whole of the surface film because the structure is not spherical, which it should be if the tension were uniform. This view is supported by the fact that potassium salts which are present in the doubly-refractive discs are localized or condensed at the ends of their longitudinal axes, a fact I hold due to the Gibbs-Thomson principle, according to which condensation of salts and other

solutes in a system is effected where the surface tension is lower than elsewhere. This indicates that the surface tension is lower at the ends of the discs than on their lateral or longitudinal surfaces. When the discs contract they tend to become spherical and can be seen to assume a greater curvature on their longitudinal surface, a result which would indicate that either the terminal faces develop a greater surface tension or the longitudinal surface film suffers a decrease. I am inclined to regard the latter as the correct interpretation.

How this decrease is brought about is another question. If the nerve impulse is merely a change of potential travelling along a nerve, its arrival at the surface of the fibril would diminish immediately and for a moment the attraction between the molecules on which surface tension of the superficial film depends, each sarcolemmal disc would become more spherical and the muscle would shorten. The diminution would develop, when initiated, in less than one-tenth thousandth of a second and it would continue as long as the charge remained in the longitudinal surface of the disc.

One can, with this explanation, account for the other phenomena of contraction. The breakdown of the lactic acid precursor is one of these. It is an observed fact that chemical action at interfaces is more intense than throughout the systems forming the interfaces, especially when the tension of the latter is lowered. A momentary diminution of the surface tension on the longitudinal surface of the sarcolemmal discs would tend to promote chemical change and this would possibly involve the breakdown of the lactic acid precursor. On the reestablishment of the surface tension in its original strength the reverse chemical action would develop, perhaps at the expense of the heat of the breakdown process or of the combustion of other constituents of the sarcolemmas or sarcoplasm, or of both the sarcolemmas and sarcoplasm.

The inequality of surface tension in the sarcolemmal disc must constantly involve the expenditure of energy, energy set free in the metabolic processes which go on in the resting muscle. In the resting muscle the repair or restorative processes keep pace with those of the breakdown which are accentuated in the contracting muscle while in the relaxing the restorative predominate. It may be that the superficial molecules are formed of the lactic acid precursor.

This explanation of the origin of muscular energy has the merit of being consistent with the motor function in amoeboid movement of protoplasm, in cilia, and in the contractile stalk of *Vorticella* which is generally accepted as a surface tension effect. It falls into line with the view that in the interior as well as on the exterior of living matter evolution has been at work without a break in continuity from the simplest form to the most complex and that everything in the highest form of life is potentially in the lowest. Surface tension is the force which in the lower organisms is the sole factor in the motor function. Can we bring ourselves to regard it as a lost property in highly specialized structure like muscle fibre and accept the contractility of the latter as due to an *ad hoc* principle exemplified nowhere else than in striated muscle fibre? The free energy on the surface of living matter or of the interfaces between its different parts, is the most readily available of all the energy in such a system and the evolutionary principle must have developed out of that free energy a force which in its highest or most specialized form is as remote from the simple surface tension of a mass of primitive sarcode only as the steam engine is from the steam kettle.

It, of course, may be urged that in the final analysis the rational conception of muscle as a structure whose function is to produce mechanical work involves the thermodynamic principle. I am not inclined to deny that, for the point of view must depend, in this case, on the definition of the terms used. It can be said that the bent steel blade is a part of a thermodynamic engine because it can produce work from the energy stored up in the elastic strain but derived originally from heat energy employed in bending the blade. The high surface tension on the lateral or longitudinal surface of each sarcous element is like the energy stored in the bent steel blade and if it could be proved to be derived directly from the kinetic energy derived from metabolism in each sarcous disc the latter would be a heat engine. It would, however, be otherwise if the energy of surface tension were only remotely, not immediately, of kinetic origin. If immediately of chemical origin does the second law of thermodynamics apply here? In a Daniel cell, which may be looked upon as a chemical engine, practically all the energy liberated appears as electrical energy and as much of it as 90 per cent may be converted into

mechanical energy and could we get rid of friction theoretically the whole of the original energy liberated could be so transformed To bring the cell back to its initial state an equivalent amount of electrical energy must be sent into the cell in the opposite direction and that amount may be produced by a heat engine turning a dynamo The cell is, therefore, at least one degree removed from a thermodynamic machine and may be classed as a chemical engine In that respect the sarcous disc is, I hold, also a chemical engine

If, consequently, surface tension is the dominant factor in the production of muscular contraction muscle is not a heat engine but a chemodynamic one The advantage of this point of view over that of the thermodynamic one lies in the fact that the theory concerned seems, in the language of the pragmatist, "to work" and it enables us to avoid labored explanations of the origin of muscular energy involving the cult of the ion and the semi-permeable membrane, or attributing thaumaturgic and inscrutable properties to colloids

NOTE

Attempts have been made to estimate the total surface energy available in muscle, based on a postulated value of the surface tension of protoplasm What this latter is is not known but Bernstein assumes that it is approximately that of oil in contact with water, that is 22 dynes per centimeter He assumes also that when muscle contracts the tension is increased in order to yield the absolute force of muscle and the energy to overcome the elasticity of muscle What the increase must be depends on the degree of fineness of the fibrils If each of the latter has a diameter of 19.988×10^{-5} cm ($= 2\mu$) and the force is 3000 grams the surface tension in contraction is 326 dynes per centimeter and that of the resting fibril would be 22 dynes per centimeter With fibrils having a diameter of 2×10^{-5} cm ($= 0.2\mu$) the surface tension of contraction would be 36 dynes per centimeter or an increase of 14 dynes above the postulated surface tension value of resting muscle fibrils

The firmness of the muscle fibrils is in itself an indication that their surface tension must be greater than that of oil in contact with water Further, as Jensen has pointed out (*Anal. Hefte*, xxvii, p. 842), a thread measuring 1 mm in diameter formed of the plasmodium of *Chondrioderma*, a Myxomycete, may, when it is in the dense condition, bear up a weight of nearly a gram If the force engaged is surface tension it would amount to about 6000 dynes per centimeter If the threads were more fluid there would be a decrease in this value but even at one-fiftieth dilution the tension would be 120 dynes as compared with 73 dynes for water

In *Orbitaltes*, a marine loraminifer, the surface tension of its cytoplasm against water is 16 dynes. The cytoplasm in this form is very fluid and mobile and is, therefore, in contrast in this respect with the plasmodia of Myxomycetes and very possibly also with the material constituting muscle fibrils and the sarcoplasm about them.

It is not improbable, therefore, that surface tension may be very high in some forms of living matter and very low in others and, consequently, estimates of the surface energy derived from a muscle in contraction, based on the supposition that its fibrils have a surface tension as low as that of oil in contact with water, are, though interesting, far from being of assistance to us in attempting to reach a solution of the problems involved.

ABSTRACT OF SCIENTIFIC PROCEEDINGS

THE EXCRETION OF PURINE CATABOLITES IN SUNDRY TYPES OF MAMMALIA

BY MAURICE H. GIVENS AND ANDREW HUNTER

(From the Department of Physiology and Biochemistry, Cornell University Medical College, Ithaca, N. Y.)

Allantoin has been isolated from, and shown to be a regular constituent of, the urine of the rabbit, horse, pig, cow, dog, cat, coyote, monkey, and man. To this list we can now add the opossum, porcupine, guinea-pig, sheep, and raccoon. In the case of the opossum, guinea-pig, sheep, coyote, and monkey we have collected a number of data bearing upon the extent of its daily excretion in starvation or on a purine-free diet, and upon the relation of the same to the simultaneous excretion of uric acid and purine bases. Our results in regard to the latter point are summarized in the following table, in which, for the sake of comparison, we have included (in brackets) a variety of related data from other sources.

ORDER	SPECIES	PER CENT OF TOTAL ALLANTOIN PURINE NITROGEN		
		Allantoin	Uric acid	Purine bases
Marsupialia	Opossum	73	21	6
Rodentia	(Rabbit)	94	6	
	Guinea-pig	93	5	2
Ungulata	(Horse)	79	21	
	(Pig)	89	2	9
	Sheep	72	12	16
	(Dog)	97	2	1
Carnivora	(Cat)	97	3	
	Coyote	96	0	4
	Monkey	74	0	26
Primates	(Man)	2	90	8

While in every case but that of man allantoin appears as the principal product of purine catabolism, it is apparent that its rôle is decidedly more prominent in some species than in others. So far as they go, the figures suggest that for each order there is a characteristic "allantoin ratio." Its values are such as to indicate that uricolytic power is greatest in carnivores, nearly as great in rodents, decidedly less in ungulates and marsupials, and practically absent in man. A strict evolutionary sequence is not apparent, although the extension of the investigation to other species and orders may reveal one. The work is being continued from this point of view.

STUDIES OF THE EXCRETION OF ACID

By LAWRENCE J. HENDERSON AND WALTER W. PALMER¹

(From the Chemical Laboratory, Massachusetts General Hospital)

In experimental studies arising from the views expressed in an earlier paper by one of us,² a large number of observations upon the excretion of acid in urine have been made.

The concentration of ionized hydrogen has been estimated in about 2500 samples of urine from about 500 individuals, of whom about one-third were normal. The normal mean appears to be very nearly 1×10^{-6} N with a range of reaction from 3×10^{-8} N to 1×10^{-5} N. The mean in pathological cases is often high, and in many instances is 5×10^{-6} N, or higher, with a range from 3×10^{-8} N to 2×10^{-5} N.

We have never found a significant diminution of acidity in pathological cases, nor in any case, normal or pathological, a degree of alkalinity greater than that of blood, save after the administration of alkali.

We have confirmed the observations of Sellards³ upon the action of ingested sodium bicarbonate upon the reaction of the urine and have found a variety of cases in which large quantities of alkali were without effect upon the hydrogen ion concentration.

¹ Henry P. Walcott Fellow in Clinical Medicine, Harvard Medical School.

² L. J. Henderson. A Critical Study of the Process of Acid Excretion, *Journ. Biol. Chem.*, ix, p. 403, 1911.

³ A. W. Sellards. The Determination of Equilibrium in the Human Body between Acids and Bases with especial reference to Acidosis and Nephropathies, *Bull. Johns Hopkins Hosp.*, xxiii, p. 289, 1912.

Further, we find that such cases, when once the urine has been made alkaline, upon discontinuing alkali until the urine is once more acid, respond to alkali in a normal manner. This observation leads us to believe that Sellards's term "tolerance" is inexact, that the phenomenon is due to a drain of alkali from the body and is, in fact, a real test for the condition of acidosis. These observations extend to a much larger variety of cases, in which more accurate estimations of hydrogen ion concentration were made, than have been observed by Sellards, and were made before we were aware of his work.

We have also studied the quantity of acid excreted in the urine (excess of acid plus ammonia) in a large number of cases. The two fractions of acid excretion appear to vary independently, though they are more nearly parallel in normal cases, in which the two moieties are likely to be nearly equal.

The ammonia appears to be an index of the degree of acidosis only in those cases where β -oxybutyric acid is produced.

The relation between hydrogen ion concentration and total quantity of acid excreted appears to provide an index of the efficiency of the kidney in carrying out the important process of acid excretion, this "functional test" possesses the advantage that it involves no experimental interference.

We have reached the conclusion that mild states of acidosis are far more common than has been suspected, and that the therapeutic use of alkali in small quantities (until the urine reaches the reaction of blood) is often desirable.

None of our observations appear to afford support for the views of Martin Fischer¹ on the cause of nephritis.

ON THE UTILIZATION OF AMMONIA NITROGEN IN THE PROTEIN METABOLISM

BY A. E. TAYLOR AND A. I. RINGER

(From the Department of Physiological Chemistry of the University of Pennsylvania, Philadelphia, Pa.)

In a series of communications by Grafe and Abderhalden in the *Zeitschrift für physiologische Chemie*, they showed that animals receiving 100 to 130 calories per kilo of body weight in the form

¹ M. H. Fischer, *Nephritis*, New York, 1912.

of carbohydrates and fats, when given ammonium citrate, carbonate or acetate, were able to retain the nitrogen to a very considerable extent. In Grafe's experiment the animals were kept in nitrogenous equilibrium with ammonium citrate.

The experiments reported here fully confirm the findings of Grafe and Abderhalden, and also show that starving and diabetic animals may retain a considerable part of the nitrogen ingested (ammonium carbonate).

THE DETERMINATION OF ACETONE BODIES IN BLOOD AND TISSUES BY MICRO METHODS

By W. McKIM MARRIOTT

(From the Laboratories of Biological Chemistry of Washington University Medical School, St. Louis, Mo.)

Determinations of acetone, diacetic acid, and β -oxybutyric acid are made on from 2-5 cc. of blood drawn with a syringe directly from the veins of living animals or patients. Acetone, preformed and from diacetic acid, is distilled off and determined by the degree of turbidity it occasions in an alkaline mercuric silver cyanide solution. Comparisons of turbidity are made in the nephelometer of Richards.

β -Oxybutyric acid is determined by oxidation to acetone according to the Shaffer method, after removal of blood proteins and sugar. The acetone found is determined as above.

THE COMPRESSION OF THE LUNGS BY INERT GASES *

By HUGH McGUIGAN AND I. C. BLCHT.

(From the Laboratory of Pharmacology of the Northwestern University Medical School.)

The compression of the lung by inert gas has been used in the past in the treatment of tuberculosis, with reported good results. This mode of treatment appears to be coming into vogue again. Our work, carried out on dogs, was undertaken primarily to determine the influence of the compression on the nutrition of the lung and secondarily, to determine the rate of absorption of the

gas (N and H) from the pleural cavity. So far no untoward result has been found on the sound tissue. The gases are absorbed rapidly and after a few days—about five—all is gone, and the lung again fully inflated, without any apparent injury to the tissue. Repeated injections must be made if the lung is to be held in the collapsed condition. The experiments are still in progress.

THE PHYSICO-CHEMICAL BASIS OF STRIATED MUSCLE CONTRACTION II SURFACE TENSION

By WILLIAM N. BERG

(Washington, D. C.)

In the following calculations, two quantities are calculated and then compared (1) the energy liberated in a working muscle through increase of surface tension times diminution of area of contractile units, and (2) the external work done in lifting a weight a known distance.

Assume that in 1 cc of muscle a right section contains 62 million rods and that there are 800 such layers, making a total of very nearly 5×10^{10} rods in 1 cc of muscle. The lateral area of a rod diminishes from $4.8 \mu^2$ when relaxed to $2.8 \mu^2$ when contracted, giving a total diminution of nearly 1000 cm^2 in 1 cc of muscle which shortens by 0.24 cm. at the same time, i. e., $800 \times 3 \mu$.

Assume the surface tension on the lateral rod surfaces to vary from that of pure acetic acid, $23 \frac{\text{dynes}}{\text{cm}}$, when relaxed, to that of saturated sodium chloride solution, $85 \frac{\text{dynes}}{\text{cm}}$, when contracted.

Since

$$\begin{array}{lcl} \text{Surface energy liberated} & = & \text{diminution in area} \times \text{surface tension} \\ \text{(ergs)} & & \text{(cm}^2\text{)} \quad \left(\frac{\text{dynes}}{\text{cm}} \right) \end{array}$$

the energy liberated is, at most, 85,000 ergs. It is almost certainly less.

The energy (ergs) expended in lifting a mass of W grams through the distance D (cm) is $W \times D \times 981$ ergs, since 1 gram (force) equals 981 dynes. Therefore in the above instance, the

85,000 ergs would lift $\frac{85,000}{0.21 \times 981} = 361$ grams, a weight that is by far too small

It is difficult to understand how surface tension can cause isometric tetanus

ON THE INFLUENCE OF PREGNANCY ON THE CYCLIC CHANGES IN THE UTERUS

By LEO LOEB

(From the Department of Pathology, Barnard Free Skin and Cancer Hospital,
St. Louis)

In previous investigations I have shown that cuts made into the uterine wall or foreign bodies introduced into the lumen of the uterus of the guinea pig about four to eight days after ovulation will lead to the formation of placentomata. I have furthermore shown that extirpation of the corpora lutea at an early stage after ovulation will prevent the formation of the placentomata. In another series of experiments I showed that extirpation of the corpora lutea within the first week after ovulation leads to a marked decrease in the length of the period of the sexual cycle in the guinea pig. The corpora lutea inhibit, therefore, the rupture of the graafian follicles. In our new investigations we examined the changes which take place in the uterine wall of the guinea pig during the different stages of the sexual period. There is a periodic change in the activity and in the morphology of the mucosa which corresponds to the various phases of the sexual cycle. At the time of ovulation, changes set in in the epithelial structures of the uterus and they are soon followed by changes in the connective tissue and blood vessels. With each new ovulation a new cycle of these changes begins. Now I found that if through early extirpation of the corpora lutea a new ovulation is accelerated simultaneously with the new ovulation a new cycle of changes sets in in the uterine wall. In order to demonstrate this fact it is necessary to ligate the fallopian tubes within the first two days after ovulation in order to prevent pregnancy from taking place. If we then examine about sixteen to twenty days after the first ovulation ovaries and uterine wall, we find new corpora lutea to have formed in the former and the cyclic changes

to have set in in the uterine wall. If, however, we ligate only one horn of the uterus, and permit pregnancy to develop in the other horn, we find upon examination of the ovaries, and of the uterine mucosa sixteen to twenty days after the previous ovulation, and after an early excision of the corpora lutea the premature ovulation in the ovaries to have taken place in a similar manner as without simultaneous pregnancy, while in the uterine wall the setting in of the new cycle has been prevented through the presence of an embryo or of a placenta in the horn of the other side. We see, therefore, that although pregnancy does not prevent the early ovulation after previous excision of the corpora lutea it prevents the setting in of a new cycle in the uterine mucosa. Pregnancy exerts, therefore, an inhibiting influence on the cyclic changes of the uterine wall, while it does not affect the early ovulation taking place after excision of the corpora lutea. Whether this inhibiting effect of pregnancy on the development of the sexual cycle is accomplished through a chemical or a nervous mechanism, or through a combination of both, cannot be decided at the present time, although it appears more probable that chemical factors play a decisive rôle in this mechanism. There is one further conclusion concerning the factors influencing the sexual cycle, to which I would like to call attention. The corpus luteum calls forth directly only certain phases in the cyclic changes in the uterine wall in the guinea pig, namely, the proliferative changes in the connective tissue of the mucosa while other changes, especially the first changes, affecting the epithelial cells of the mucosa are not directly dependent on the function of the corpus luteum. This latter conclusion is based on the observation that some of the cyclic changes in the uterine wall precede the formation of the corpus luteum.

ON THE MECHANISM OF STIMULATION BY OXYGEN WANT

By H. S. GASSER AND A. S. LOEVENHART

(From the Laboratory of Pharmacology of the University of Wisconsin)

We have demonstrated that carbon monoxide, when administered in certain dosage, causes a rise in blood pressure. We believe this to be due to stimulation of the vasomotor center. We believe the failure of other authors to observe this stimulation

to be due to the fact that too much of the gas was administered and that the second stage of the action, namely, depression was noted

We find that the medullary centers preserve the same order in regard to their sensitiveness to hydrocyanic acid and carbon monoxide as has been shown for anemia, that is, the respiratory center is the most sensitive, the vasomotor center is intermediate, and the cardio-inhibitory center is the least sensitive. We find that the latent period of the response of the respiratory and vasomotor centers to decreased oxidation, as brought about by means of the administration of hydrocyanic acid and carbon monoxide, is so short (on the average the respiratory center is stimulated in 4 seconds by hydrocyanic acid and in 6 seconds by carbon monoxide) that the stimulation cannot be due to abnormal metabolic products accumulated during decreased oxidation. Our conclusions may be stated as follows:

1. Oxygen want stimulates the cells of the respiratory, vasomotor, and cardio-inhibitory centers by decreasing oxidative processes within the cells of these centers.

2. Decreased oxidation does not bring about stimulation through accumulation of abnormal metabolic products.

3. We believe that decreased oxidation *per se* results in stimulation. Under conditions of decreased oxidation, it seems probable that other processes not directly requiring oxygen are increased, and that functional activity is the external expression of these latter processes.

FEEDING EXPERIMENTS RELATING TO THE NUTRITIVE VALUE OF THE PROTEINS OF MAIZE

By THOMAS B. OSBORNE AND LAFAYETTE B. MENDEL

(*From the Laboratories of the Connecticut Agricultural Experiment Station
and the Sheffield Laboratory of Physiological Chemistry,
Yale University, New Haven, Connecticut*)

According to the data now available more than one-half of the proteins of maize consists of zein, a type exhibiting such unique chemical and physical characters as to make probable that its nutritive properties differ from those of other proteins. About one-third of the proteins consists of maize glutelin, insoluble in

neutral solvents, extracted from the seed only by dilute alkalis, and yielding all the amino-acids characteristic for most other proteins. When zein forms the sole protein of the dietary, rats speedily decline in weight despite an apparently sufficient food intake. The decline is not due to digestive failure, for the food can be made adequate for maintenance over a considerable period by the addition to the food of tryptophane (which is missing among the decomposition products of zein). When half of the zein is replaced by another protein, such as casein, lactalbumin, edestin, or maize glutelin, nutritive decline can be checked. The proportion necessary varies with the different proteins. In contrast with zein, which lacks tryptophane, lysine, and glycocholic acid, gliadin, which is deficient in the last two only, suffices for maintenance without growth. Maize glutelin is adequate for normal growth. Foods containing equal parts of zein and maize glutelin promote nearly normal rate of growth, this applies likewise to the natural mixture of them as exhibited in so-called corn gluten. This material affords an opportunity to study the nutritive value of the two maize proteins before they have been subjected to any chemical manipulations. Animals kept on foods containing additions of both tryptophane and lysine to zein have been maintained over long periods of time. These observations all emphasize the extreme importance of tryptophane in successful dietaries. It also appears probable that the deficiency observed in the practical feeding of corn meal is explained in good part by the unique chemical constitution of zein which forms so large a part of its nitrogenous components.

INTESTINAL OBSTRUCTION. STUDY OF A TOXIC SUBSTANCE PRESENT IN THE INTESTINAL MUCOSA

By G. H. WHIPPLE

(From the Pathological Laboratory, Johns Hopkins Medical School.)

Dogs with closed washed duodenal loops (ligatures just below pancreatic duct and just beyond duodeno-jejunal junction, with gastro-enterostomy) die in about two days with low blood pressure and temperature, vomiting and diarrhoea, the symptoms of shock seen in high obstruction and volvulus. The mucosa of these closed loops may be intact and normal except for congestion,

yet a toxic substance can be isolated from it. Normal mucosa gives no such substance. This toxic substance is active when given intravenously, intraperitoneally and subcutaneously, but is not absorbed from the normal intestine. It causes a profound fall in blood pressure and temperature, profuse vomiting and diarrhoea and collapse. Death follows in two to twenty hours with the general picture of fatal anaphylaxis. Autopsy shows a remarkable splanchnic congestion most marked in the intestinal mucosa which may be deep purple in color. Destruction of the mucosa by sodium fluoride is followed by death from peritonitis, but the distended loop contains no toxic substance, indicating that the mucosa is essential to the elaboration of this substance. Dogs injected with sublethal doses of this toxin are resistant to later injections and if a closed loop is produced in such an animal it may live six days instead of two to three days.

THE INFLUENCE OF THE PLANE OF PROTEIN INTAKE ON NITROGEN RETENTION IN THE PIG

By E. V. McCOLLUM

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin)

Experiments have been carried out in the following manner in order to compare the values of the nitrogen of the wheat and maize kernels, and of rolled oats for growth in the pig. The animals, weighing from 45-50 pounds, were placed in metabolism cages and fed starch during ten days, and the creatinine nitrogen output determined daily. The average amount was assumed to represent 18.5 per cent of the nitrogen from endogenous metabolism⁶. The animals were then fed on individual grains, or with the grains plus a commercial, protein-rich by-product, so that the ration was derived in all cases from a single grain source. The nitrogen intake was adjusted at levels of 5, 7.5, 10, 15 and 20 times the endogenous requirement, in various experiments. These levels correspond approximately to 9.5, 13, 18, 28 and 37 per cent of protein in the ration. Feeding was continued about sixty days, and was followed by a ten-day period on starch. In all

⁶ McCollum *Amer Journ of Physiol*, xxix, p 210, 1911

cases the energy value of the rations was 100 calories per kilogram of pig per day

The retention of nitrogen at all levels except 7.5 times the endogenous level, was in close agreement in all experiments, whether derived from wheat, oats or maize. At 5 times maintenance about 10 per cent of the ingested nitrogen was retained. At 7.5 times, the retention varied from 12-17 per cent in different experiments, due doubtless to the inferiority of certain of the pigs. At levels of 10, 15 and 20 times the endogenous requirement, the retention was 21-24 per cent of the ingested nitrogen, and was therefore independent of the source or plane of intake.

In one experiment still in progress, a pig of 45 pounds initial weight consumed 57.4 grams of nitrogen per day, derived from a mixture of wheat embryo and wheat gluten, during thirty-nine days. It is apparent that he has retained about 25 per cent of the ingested nitrogen.

Further work is in progress at the plane of 7.5 times the endogenous requirement, and at high planes of protein intake.

ENZYME SYNTHESSES

By H. C. BRADLEY

(From the Laboratory of Physiology of the University of Wisconsin)

This work is an extension of that reported upon last year. A wide survey of tissues both vertebrate and invertebrate fails to show any correlation between the lipase activity of a tissue and its fatty metabolism as expressed by its ability to store up fat. In the same way there seems to be no correlation between the diastase of tissues and their glycogen content in period of nutritional abundance. Certain invertebrate tissues rich in glycogen were found to exhibit but a trace or no diastatic activity. In plants a certain amount of evidence can be found in favor of the hypothesis that diastase and lipase produce the synthesis of starch and esters. It seems probable that in the plant the storage of starch in seeds and tubers is the result of diastatic synthesis, and that in some seeds and fruits the development of oils and esters is accomplished through the agency of lipase. No evidence could be found of a lactose-splitting enzyme in the active mammary glands from a number of animals. It is probable that lactase is not involved in the synthesis of lactose.

FURTHER OBSERVATIONS ON THE INFLUENCE OF CAFFEINE ON CREATINE AND CREATININE METABOLISM⁷

By W. SALANT AND J. B. RIEGER

(From the Laboratory of Pharmacology, Bureau of Chemistry, U. S.
Department of Agriculture, Washington, D. C.)

The excretion of creatinine and creatine was studied in well fed and in fasting animals. In rabbits which were fed oats, a moderate increase of creatine was observed after the subcutaneous administration of doses of 100 and 150 mgs per kilo. When food was withdrawn the amount of creatine was increased enormously in some rabbits. Creatinine was only slightly increased in the same subjects. The subcutaneous injection of 50 mgs per kilo also increased the output of creatine in fasting rabbits as well as in animals which received oats. In some experiments, however, neither creatine nor creatinine was affected by the administration of caffeine even when food was withheld. Experiments on dogs with doses of 50-200 mgs per kilo were negative. Creatine and creatinine metabolism was not affected in well fed or in fasting dogs by caffeine whether given by mouth or subcutaneously.

A NEW METHOD OF DETERMINING VALENCE FROM THE MOLECULAR COHESION

By ALBERT P. MATHEWS

(From the Laboratory of Physiology, University of Chicago)

A new method was found of computing " a " of van der Waals' equation from the law of Eötvös and the formula of Thomas Young. The values thus obtained are in general similar to those obtained by the ordinary methods, but for simple substances are in general higher. If " a " be expressed as $N^2 M^2 K$, N being the number of molecules in the volume, V , then if the expression of $\frac{a}{V^2}$, representing molecular cohesion in van der Waals' equation, be divided in both the numerator and denominator by N^2 , we have $\frac{M^2 K}{v^2}$, the molecular cohesive pressure, since v is the space

⁷Read by title

at the disposal of a single molecule. The factor M I have called the mass of molecular cohesion. It was found by trial that the factor M^2K was a function of the molecular weight and the number of valences in the molecule, or $M^2K = C (\text{Mol Wt} \times \text{Val})^{2/3}$, where C is a constant equal to 2.98×10^{-37} , if " a " is expressed in absolute units. This gives a means of computing valence when the critical data are known. By this method it was shown that most substances had the number of valences ordinarily ascribed to them, but that chlorine was always trivalent, argon was monovalent, nitrogen, as a gas, was monovalent and oxygen, as an element was monovalent, each molecule having two valences. By this method acetylene is shown to have ten valences, it is not, therefore, acetylidene. The trivalency of chlorine is indicated also by the optical method of determining valence and by the diamagnetic method. Various changes in graphic formulae will be necessitated by the changes of valence indicated by this method. The probable theoretical significance of this relationship of valence to cohesion is that cohesion is allied to magnetism and that the two kinds of electrons in a molecule, atomic and valence, differ in their freedom so that they can not be summed.

THE ENTRANCE OF IODINE INTO DISEASED TISSUES

By H. GIDEON WELLS AND O. F. HEDENBURG

(From the Otho S. A. Sprague Memorial Institute and the University of Chicago)

A systematic consideration of the chemotherapy of tuberculosis rests upon an investigation of the permeability of both the tubercle bacillus and the tuberculous lesion for chemical substances of different characters. It is shown that compounds of iodine injected into tuberculous animals enter glandular tubercles with readiness, so that the proportion of iodine in such tubercles is usually greater than it is in most other tissues except the kidney, furthermore it is greater in the caseous contents than in the cellular peripheries of the tubercles. Tuberculous eyes also contain much more iodine than the normal mates. This property is shown not to depend upon any specific character of the tubercle itself, for other necrotic tissues also contain more iodine than normal tissues. The explanation offered is that normal cells are

not perfectly permeable to iodides (except perhaps kidney cells) and lose this impermeability or semipermeability when killed or injured, thus becoming entirely permeable for crystalloids present in the surrounding fluids. As the iodine content of the blood increases and decreases with absorption and elimination, so varies the iodine in the necrotic area, whether tuberculous or otherwise, indicating an absence of any chemical or physical binding of the iodine in such areas. A simple inert colloid, agar, implanted in the tissues, behaves in quite the same way.

Egg albumen injected into tuberculous pigs is found, by means of the anaphylaxis reaction, to penetrate the avascular tubercles but little if at all, even when present in the blood in large amounts. This fits with the hypothesis that the passage of iodine from the blood into the tubercles is a purely physical matter, the crystalloidal iodine compounds diffusing through the inert colloidal solution of a necrotic area practically unimpeded, while the colloidal egg albumen, according to the law of colloidal diffusion, is practically unable to diffuse through such a colloidal solution.

No evidence could be found of any tendency for iodine compounds of whatever nature to accumulate in tubercles or other necrotic areas, or to persist in such areas when disappearing from the normal tissues and the blood.

Exudates contain approximately the same proportion of iodine as the blood of the same animals, and hence any area with inflammatory edema and congestion will commonly show more iodine than normal tissues, although not usually more than the blood. No evidence was found of any specific entrance or fixation of iodine in inflammatory exudates. The iodine is distributed about alike in the fluid and solid portions of the exudate, indicating simple diffusion. Of normal tissues only the kidney seems to contain approximately as much iodine as the blood of the same animal. This may have some bearing upon its excretory function, since it indicates a greater permeability of renal cells than of other gland cells for iodides.

SARCOLACTIC ACID AND THE THEORY OF DIABETES

By R T WOODYATT

(From the Clinical Laboratory of the Sprague Memorial Institute, Rush Medical College, Chicago)

Theory proposed "The function of the internal secretion of the pancreas is to dissociate glucose and perhaps other hexoses. Its action resembles that of alkali on sugars in general. It depends upon the presence in the internal secretion of an organic base, which forms a glucosate whose dissociation is high. Dissociation of sugar in the body is essential for its oxidation, reduction, polymerization, cleavage and for its participation in a great organic equilibrium which includes glycogen, glucose, split-products of glucose (*e g*, trioses and lactic acid), other hexoses, certain amino and fatty acids (*e g*, alanine, propionic acid), protein and fat. All anomalies in pancreas diabetes are explainable by lessened glucose dissociation with the changes in oxidation and polymerization and the disturbed organic equilibrium which this implies."

Lactic acid coming from glucose is, *per se*, evidence of dissociation of glucose in the body. The weights of lactic acid (as zinc lactate) found in muscles so treated as to insure maximum survival formation ran as follows

	Grams
Normal dogs (average of 4 determinations)	0.542
Phlorhizinized dog (D N = 3.65, 1, muscle-glycogen, 0)	0.127
Phlorhizinized dog (D N = 2.8, 1, muscle-glycogen, trace)	0.227
Phlorhizinized dog (D N = 2.9, 1, muscle-glycogen, 0.22)	0.296*
Diabetes mellitus	0.077

* Intravenous glucose injection ante mortem to produce hyperglycaemia comparable to diabetes mellitus

THE PRESENCE OF AN ACID PRODUCING ENZYME IN *BACT LACTIS ACIDI**

By E G HASTINGS AND E B HART

(From the Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin)

The work of Buchner, Herzog, and others has shown the presence of an enzyme in certain lactic acid-producing bacteria that are essentially different from the organisms predominating in

*Read by title

cheese. This intracellular enzyme, which can be demonstrated only after the disintegration of the cell, forms small quantities of lactic acid from sugar. So far as is known to us, a similar enzyme has never been demonstrated in organisms of the *Bact. lactis acidii* group. The growth of these organisms on all media is so meager that it is very difficult to obtain a sufficient amount for treatment by the methods employed by Buchner. An acid-producing enzyme in the lactic acid bacteria has, however, been demonstrated by quite different methods.

It had been noted that when a sample of raw or sterilized milk in which varying numbers of lactic bacteria had been allowed to develop, and to which a preservative, as chloroform or toluol, had been added, the cells soon disappeared or at least could no longer be detected by microscopical examination. A sample of fresh raw milk and one of the same milk heated to 97°C for a short time were inoculated with a pure culture of *Bact. lactis acidii*. At varying periods in the development of acid, samples were removed from each and preserved with 3 per cent of toluol. In those treated soon after inoculation, a small number of bacteria were present while in those to which the preservative was added at a later stage in the development of acid, a much greater bacterial growth was present. If any enzymic action occurred, the samples should have shown a difference in the amount of acid formed corresponding to the amount of bacterial cells present.

The results seem to leave no doubt concerning the presence of an acid-forming enzyme in the organisms of the *Bact. lactis acidii* group that acts on the milk sugar. It might be thought that the increase in acidity was due to the production of amino-acids by a proteolytic enzyme. In this case the soluble nitrogen must be increased. That this does not occur has been shown by numerous investigations. The increase in acid, however, cannot be asserted to be due to the formation of lactic acid since, of course, no qualitative test could be made in the presence of the lactic acid in the milk at the beginning of the experiment.

THE INFLUENCE OF THE COMPOSITION AND AMOUNT OF THE MINERAL CONTENT OF THE RATION ON GROWTH

By E V McCOLLUM AND MARGUERITE DAVIS

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin)

Young rats do not grow when fed the wheat kernel alone, or the wheat kernel plus wheat gluten to give a protein content of 18 per cent, when distilled water is given. The latter ration, with salts added to make the total content of the mineral elements closely similar to that of milk powder, has produced normal growth in rats during seventy days.

Rats grow normally during seventy-five to one hundred days on a ration consisting of pure casein, 18 per cent, dextrin, agar-agar and salt mixtures giving an inorganic content closely similar to either milk or egg yolk, and on certain other salt mixtures, in about the proportions found in milk and in eggs. With the same organic ration, fed with a salt mixture giving the ration an inorganic content closely similar to that of the wheat kernel, there is a complete suspension of growth. The addition of calcium, or the subtraction of magnesium induces growth in some degree. This is true also of rations derived from wheat and wheat gluten.

We have much experimental evidence indicating that an important relation exists between the proportion of the mineral elements in the diet, to its protein content. Normal growth has been secured during seventy days on a ration of casein, 34 per cent, dextrin, agar-agar and a salt mixture giving an inorganic content similar in composition and quantity to that of dry skim milk. With the mineral content like that of egg yolk, and fed in the same proportion as found in dry egg yolk diluted to 12 per cent protein content, considerable growth has been observed. It is noteworthy that both highly acid as well as slightly alkaline mixtures lead to good results if the composition is satisfactory.

CONNECTIVE TISSUES OF LIMULUS

By H C BRADLEY

(From the Laboratory of Physiology of the University of Wisconsin)

Attached to the gill flaps of *limulus* are rods of cartilage-like tissue. Physically and histologically it resembles cartilage. Chem-

really it is a typical sclero-protein, insoluble in water and the ordinary solvents, digesting readily in a tryptic mixture but insoluble in pepsin-HCl. It contains 11.4 per cent N, 1.6 per cent $\text{NH}_2\text{-N}$, 3.1 per cent tyrosine. On hydrolysis with strong HCl it yields considerable sulphate, and a small amount of a reducing substance. All of the ordinary protein reactions are positive. It does not yield sufficient gelatin to give a positive test for that substance.

Across the ventral portion of limulus, within the carapace, extends a white fibrous mass of connective tissue connecting a large number of muscles. Its general resemblance in appearance and function to mammalian tendon is further borne out by its histological appearance. Chemically it also is a sclero-protein, free from collagen and somewhat different from the cartilage. It contains 16.0 per cent N, no ammonium N, 2.4 per cent tyrosine, no oxidized sulphur, and a trace of a reducing substance. It digests rapidly in pepsin-HCl, and very slowly in a tryptic mixture.

A NEW METHOD FOR DETECTION OF SMALL AMOUNTS OF CARBON DIOXIDE

By SHIRO TASHIRO

(From the Laboratory of Physiology, University of Chicago)

Making use of the principle which Dr. McCoy and I discovered, I have constructed two new pieces of apparatus which not only detect CO_2 in amounts as small as 0.0000001 gram, but also estimate it with quantitative accuracy. The first apparatus consists of two chambers, the upper one being used for the qualitative detection of the gas, the lower for the quantitative estimation. The second apparatus is used for the combined purposes of qualitative, quantitative and comparative estimation of CO_2 productions from various biological specimens. Unlike any other method for analysis of a gas in small quantity, the new method requires no correction nor precaution against the effects of temperature and pressure variations.

A STUDY OF THE CHEMICAL CHANGES OCCURRING IN
MEATS DURING THE PROCESS OF DRYING BY THE
VACUUM METHOD⁹

By L H DAVIS AND A D EMMETT

(From the Department of Animal Husbandry, University of Illinois)

The average percentage values for the analysis of five samples of fresh meat and of the corresponding samples of desiccated meat, prepared by Shackell's modification of the Benedict-Manning method were, respectively 26.46 and 98.21 for dry substance by the vacuum method over sulphuric acid, 26.38 and 96.55 for dry substance by heating in an air oven at 102° C, 2.77 and 10.14 for fat on the vacuum-dried samples, 3.21 and 9.70 for fat on the oven-dried samples, 3.464 and 13.124 for total nitrogen, 0.795 and 2.769 for total water soluble nitrogen, 0.420 and 1.182 for heat coagulable nitrogen, 0.021 and 0.107 for proteose nitrogen, 0.122 and 0.667 for creatine nitrogen, 6.176 and 19.711 for total soluble solid and 0.78 and 3.14 for total soluble ash.

On the dry basis (vacuum method), the average percentage composition of the fresh and desiccated meats was respectively 10.22 and 10.31 for fat, 13.138 and 13.368 for total nitrogen, 3.011 and 2.835 for total soluble nitrogen, 1.591 and 1.204 for heat coagulable nitrogen, 0.080 and 0.109 for proteose nitrogen, 21.94 and 19.96 for total soluble solids and 2.90 and 3.18 for total soluble ash.

The forms of nitrogen expressed in per cent of the total gave for the fresh and desiccated meats the following respective percentage values: the total soluble, 22.95 and 21.17, the heat coagulable, 12.13 and 8.98, and the proteose, 0.62 and 0.82.

Calculating the data for the fresh and desiccated meats to the dry basis using the two values for dry substance, the vacuum and the oven-heated—the results agree quite closely for the various constituents, the greatest differences being in the fat as was to be expected.

⁹Read by title

MUSCLE CREATINE DIALYSIS OF CREATINE FROM DOG MUSCLE ¹⁰

(PRELIMINARY REPORT)

BY H. T. LEO AND PAUL E. HOWE

(From the Laboratory of Physiological Chemistry, University of Illinois)

A study was made of the dialysis of creatine from muscle under conditions which would tend to throw light upon the form in which this substance is held in the tissues. The experiments of Urano were repeated, using dog-muscle (a) in the form of bundles, (b) ground and placed loosely or packed tightly in collodion bags. Dialysis was commenced immediately after the removal of the muscle from the animal or after it had remained for 24 hours on ice. Each preparation was dialyzed for 2, 2, 2, 12, and 24 hours against 200 cc portions of Ringer's solution, distilled water, 0.9 per cent NaCl, 1.8 per cent NaCl, 5 per cent NaCl, Ringer's solution + HCl (to make 0.13 per cent), 0.9 per cent NaCl + HCl, 0.13 per cent HCl, and 70 per cent alcohol.

The data indicate that creatine dialyzed most readily from those preparations which were in the loosely-packed, finely-divided form and least, from the more solid preparations, as the muscle bundles and the firmly-packed ground muscle. Dialysis experiments conducted with those substances which should tend to increase the rate of dialysis gave variable results. The action of HCl upon ground muscle was to cause a slower rate of diffusion, but when added to the 0.9 per cent NaCl solution an increased rate of dialysis occurred. The action of the acid upon muscle bundles caused an increase in the rate of diffusion. Alcohol appears to increase the rate of dialysis from muscle bundles but the effect is not equal to that obtained with the HCl nor with the ground muscle in salt solution. Approximately the same results were obtained with the Ringer's solution as with the 0.9 per cent NaCl solution. The hypertonic salt solutions gave a more gradual rate of dialysis, although the ultimate effect was the same. When the results from the various experiments are plotted, the form of the curve is the same (hyperbolic).

The results indicate that simple diffusion experiments do not appear to offer any definite evidence as to the manner in which creatine is held in muscle.

¹⁰Read by title

STUDIES ON SULPHUR METABOLISM I THE URINARY SULPHUR PARTITION IN VARIOUS DISEASES ¹¹

BY N STADTMÜLLER, M KAHN AND J ROSENBLOOM

(From the German Hospital, New York, and the Laboratory of Biochemistry of the University of Pittsburgh, Pittsburgh, Pa)

We have studied the urinary sulphur partition (total sulphur, sulphate-sulphur, ethereal sulphate-sulphur, inorganic sulphate-sulphur and neutral sulphur) in the following diseases

Diabetes mellitus, 10 cases, carcinoma, 13 cases, nephritis, 2 cases, pneumonia, 2 cases, lead poisoning, 2 cases, bronchial asthma, 1 case, chronic appendicitis, 2 cases, hepatic abscess, 1 case, hepatitis, 2 cases, cholelithiasis, with biliary fistula, 1 case, typhoid fever, 1 case, chronic myocarditis, 1 case, hypopituitarism, 1 case, gastroptosis and gastric dilatation, 1 case

The urine has been analyzed daily for periods varying from three days to two weeks, and we have found high proportions of neutral sulphur in all the cases of diabetes (except one) The neutral sulphur was also high in all the cases of carcinoma, in one case of nephritis and pneumonia and in the one case of hypopituitarism

THE METABOLIC END-PRODUCTS OF THE LIPOID NITROGEN OF EGG YOLK ¹¹

By E V McCOLLUM AND H STEENBOCK

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin)

A pig of 49 pounds was fed during seven days on a ration of 220 grams of dry egg yolk and 35 grams of starch per day The nitrogen intake was 11.65 grams per day Of this 0.65 gram was lipid nitrogen The total N of the urines varied from 5.029-6.098 grams From 30 to 40 per cent of the total nitrogen was removed by the Folin method for ammonia Urea¹ by the Benedict and by the Henriques and Gamble² gave fairly close agreement, and indicated that 10 per cent of the total nitrogen was present in the urine

¹¹Read by title

Of the nitrogen removed by aspiration, 5.2-16.7 per cent could not be absorbed by mercury oxide according to Erdmann's method. By this method the urines were found to contain on an average 0.2989 gram of nitrogen as substituted amines. It is evident that demethylation of substituted amines is not readily accomplished in the body of the pig.

THE OCCURRENCE OF URIC ACID IN BLOOD ¹²

By OTTO FOLIN AND W. DENIS

A NEW METHOD FOR DRYING TISSUES AND FLUIDS ¹³

By JACOB ROSENBLUM

METABOLISM OF A DWARF ¹⁴

By GRAHAM LUSK

THE FATE OF FATTY ACIDS IN DIABETIC ORGANISMS ¹⁵

By A. I. RINGER

ON THE SECRETION OF PURE ACID BY THE KIDNEY

By A. B. MACALLUM AND W. R. CAMPBELL

HYPERTROPHY AND HYPERPLASIA OF THE PARATHYROID IN BIRDS

By D. MARINE

QUANTITATIVE OXIDASE MEASUREMENTS ¹⁶

By H. H. BUNZEL

THE REGULATING FUNCTION OF AMYLASE BY THE FUNGUS GLOMERELLA ¹⁷

By HOWARD S. REED

SYMPOSIUM

SOME RECENT APPLICATIONS OF PHYSICAL CHEMISTRY IN BIOLOGY

A. B. MACALLUM —Surface tension

L. J. HENDERSON —The control of neutrality in the animal body

A. S. LOVENHART —The physical chemistry of enzyme action

¹² Published in full in *Journal of Biological Chemistry*, xiv, February, 1913

¹³ Published in full in *Journal of Biological Chemistry*, xiii, January, 1913

¹⁴ Read by title

RESEARCHES ON PURINES

ON 2,8-DIOXY-1,9-DIMETHYLPURINE AND 2-OXY-6,9-DIMETHYLPURINE

(EIGHTH PAPER¹)

By CARLO O. JOHNS

(From the Sheffield Laboratory of Yale University)

(Received for publication, December 18, 1912)

Two of the nine possible isomerides of 2,8-dioxy-dimethylpurine have been described, namely, 2,8-dioxy-3,7-dimethylpurine,² which was obtained by Fischer, and 2,8-dioxy-6,9-dimethylpurine³ The latter of these compounds, although isomeric with theobromine (VI), is not exactly analogous since one of the methyl groups is attached to carbon while in theobromine both of the methyl groups are attached to nitrogen In 2,8-dioxy-1,9-dimethylpurine (IV) both of the methyl groups are attached to nitrogen, hence one might expect it to exhibit properties comparable with those of theobromine Theobromine is a strong diuretic Professor Mendel has tested the action of 2,8-dioxy-1,9-dimethylpurine on rabbits but found that it did not produce notable diuresis⁴

2,8-Dioxy-1,9-dimethylpurine was synthesized as follows The potassium salt of 2-oxy-5-nitro-6-methylaminopyrimidine⁵ (I) was heated with methyl iodide and gave 2-oxy-3-methyl-5-nitro-6-methylaminopyrimidine (II) the constitution of which was shown by heating the methylated product with sulphuric acid

¹ *This Journal*, xii, p 91, 1912

² Emil Fischer *Ber d deutsch chem Gesellsch*, xxviii, p 2487, 1895, xxx, p 1851, 1897, xxxii, p 474 1899

³ Johns *this Journal*, vi, p 397, 1912

⁴ Reported at the fourth annual meeting of the American Society for Pharmacology and Experimental Therapeutics, Cleveland, Dec 30, 1912

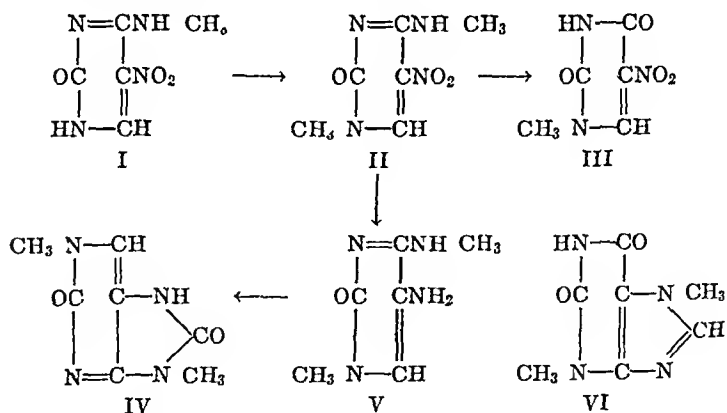
⁵ Johns *this Journal*, ix, p 164, 1911

and obtaining 2,6-dioxy 3-methyl-5-nitropyrimidine⁶ (III) When 2-oxy-3-methyl-5-nitro-6-methylaminopyrimidine was reduced with freshly precipitated ferrous hydroxide it was converted to 2-oxy-3-methyl-5-amino-6-methylaminopyrimidine (V) As might be expected, the presence of the two methyl groups rendered the compound extremely soluble It was therefore difficult to purify and after analyzing its picrate the crude base was heated with an equal weight of urea whereupon 2,8-dioxy-1,9-dimethylpurine (IV) was obtained

A new isomer of the three 2-oxy-dimethylpurines⁷ previously described has also been prepared, namely, 2-oxy-6,9-dimethylpurine (IX) This compound was obtained by heating the potassium salt of acetyl-2-oxy-4-methyl-5,6-diaminopyrimidine (VIII)

During the course of this investigation it was found that 2-oxy-4-methyl-5,6-diaminopyrimidine can easily be produced in quantity by reducing 2-oxy-4-methyl-5-nitro-6-aminopyrimidine⁸ with freshly precipitated ferrous hydroxide This method is a decided improvement on the one previously used in which aluminium amalgam was employed as the reducing agent

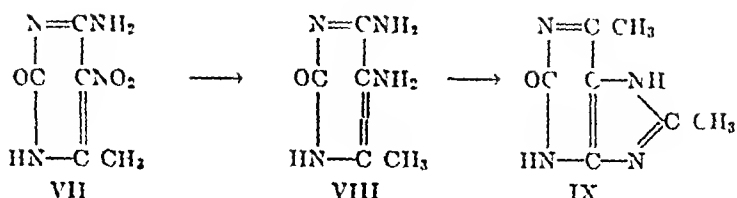
These researches will be continued



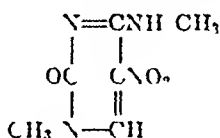
⁶ Behrend and Thurm *Ann d Chem* (Liebig), cccxviii, p 163, 1902

⁷ Julius Tafel *Ber d deutsch chem Gesellsch*, xxvii, p 3201, 1899,
 Johns this *Journal*, xii, p 91

⁸ Johns *Amer Chem Journ*, xli p 61, 1909



EXPERIMENTAL PART

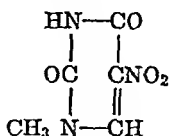
2-Oxy-5-methyl-5-nitro-6-methylaminopyrimidine,

Five grams of finely pulverized 2-oxy-5-nitro-6-methylaminopyrimidine⁹ were dissolved in 40 cc of water containing 1.9 grams of potassium hydroxide, and 5 grams of methyl iodide were then added. This mixture was heated at 100°C in a sealed tube for one hour. This treatment produced an almost colorless solution from which a bulky mass of hair-like crystals separated on cooling. A second crop was obtained by concentrating the mother liquor. A trace of free iodine was removed by making the solution alkaline with sodium hydroxide. The crystals thus obtained were readily soluble in hot and moderately soluble in cold water. They were easily soluble in cold chloroform, moderately soluble in hot alcohol and slightly soluble in boiling benzene. They melted to a colorless oil at 203°C. The yield was about 50 per cent of theory.

	Calculated for $\text{C}_6\text{H}_8\text{O}_2\text{N}_4$	Found
N	30.43	30.12

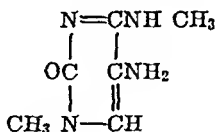
All of the nitropyrimidine used in the above experiment had reacted as none of it was recovered unaltered. An exceedingly soluble by-product was formed. This contained iodine that was not removed by treatment with alkalis. An investigation of this by-product was deferred until more material can be obtained.

⁹ Johns, this *Journal*, ix, p 164, 1911

2,6-Dioxy-3-methyl-5-nitropyrimidine,

In order to determine the position that the methyl group had occupied in the alkylation of 2-oxy-5-nitro-6-methylaminopyrimidine the methylamino group in position 6 was removed by hydrolysis as follows. One half gram of the methylated product was dissolved in 10 cc of 25 per cent sulphuric acid and the solution was heated in a sealed tube at 160°–170° C for two hours. On standing over night the resulting solution yielded a crop of beautiful crystals. These melted at 255° C and when mixed with a pure sample of 2,6-dioxy-3-methyl-5-nitropyrimidine¹⁰ the melting point was not lowered. Hence alkylation had occurred in position 3 in the pyrimidine ring. The portion used for analysis was dried at 115° C to remove the water of crystallization.

	Calculated for $\text{C}_4\text{H}_5\text{O}_4\text{N}_3$	Found
N	24.56	24.55

2-Oxy-3-methyl-5-amino-6-methylaminopyrimidine,

Five grams of 2-oxy-3-methyl-5-nitro-6-methylaminopyrimidine were dissolved in 200 cc of a mixture of equal parts of water and concentrated ammonia. Fifty grams of ferrous sulphate contained in 100 cc of water were added. Reduction took place rapidly as was shown by the immediate formation of ferric hydroxide. A solution of 62 grams of crystallized barium hydroxide in hot water was then added to precipitate the SO_4 and a small excess of baryta was precipitated by means of ammonium carbonate. The mixture was heated on a steam bath for an hour and then

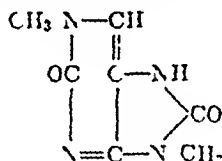
¹⁰ Behrend and Thurm *loc cit*

filtered by suction. The filtrate was evaporated to a syrup which hardened on cooling. The substance thus obtained was exceedingly soluble in water or alcohol but did not dissolve in benzene or ether. On account of the difficulties encountered in attempting to purify this base it was used in the crude state for the preparation of 2,8-dioxy-1,9-dimethylpurine.

A picrate of 2-oxy-3-methyl-5-amino-6-methylaminopyrimidine was made by mixing a rather concentrated solution of the crude base with a saturated aqueous solution of picric acid. The picrate separated slowly in the form of prisms that were about five times as long as they were thick. They were very soluble in hot water. They melted with decomposition at about 200°C.

N	Calculated for		Found
	$C_{11}H_{10}ON_4$	$C_{11}H_9(NO_3)_3O_4H$	
	25.61		26.00

2,8-Dioxy-1,9-dimethylpurine,

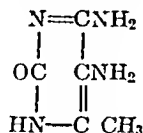


The crude 2-oxy-3-methyl-5-amino-6-methylaminopyrimidine which was obtained in the preceding experiment was mixed with an equal weight of urea and the mixture was heated for an hour at 180°C in an oil bath. The residue was taken up in hot dilute ammonia and the solution was decolorized by means of blood coal. On boiling off most of the ammonia and acidifying with acetic acid the purine crystallized rapidly in the form of small plates. A second crop was obtained by concentrating the mother liquor to a small volume. The yield of pure material was about 50 per cent of theory when calculated on the basis of the nitropyrimidine used. The purine dissolved in less than 40 parts of boiling water and crystallized in small irregular plates that did not melt or char at 320°C. It dissolved readily in ammonia and was precipitated by acetic acid. It was slightly soluble in boiling alcohol but almost insoluble in boiling benzene. It did not give a murcic reaction.

0.1869 gram of substance gave 0.0740 gram of H_2O and 0.3174 gram of CO_2 .

	Calculated for $C_7H_8O_2N_4$	Found
C	46.66	46.33
H	4.44	4.39
N	31.11	31.10

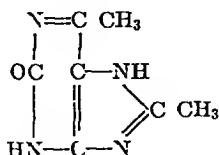
2-Oxy-4-methyl-5,6-diaminopyrimidine,¹¹



This compound has been described in a previous communication from this laboratory. It was first obtained by reducing 2-oxy-4-methyl-5-nitro-6-aminopyrimidine by means of aluminium amalgam. This method was laborious as only small quantities could be reduced at one time and the yields were low. It has been found that larger quantities can be reduced smoothly by the use of freshly precipitated ferrous hydroxide.

Twenty-two grams of 2-oxy-4-methyl-5-nitro-6-aminopyrimidine were dissolved in a mixture of 200 cc of water and 200 cc of concentrated ammonia. To this solution were then added 264 grams of crystallized ferrous sulphate in a small volume of hot water. Reduction took place rapidly. The SO_4 was precipitated by adding a hot solution of 300 grams of crystallized barium hydroxide and removing the excess of baryta with ammonium carbonate. After filtering by suction, washing with hot water, and concentrating the filtrate the diaminopyrimidine was obtained in a crystalline form. The yield was 80 per cent of theory.

2-Oxy-6,8-dimethylpurine,



¹¹ Johns *Amer Chem Journ*, xl, p 61, 1909

Five grams of 2-oxo-4-methyl-5,6-diaminopyrimidine were suspended in 25 cc of acetic anhydride and the mixture was heated in an evaporating dish on a steam bath. Complete solution did not occur as the crystals of the acetyl compound separated rapidly. The mixture was evaporated to dryness and the last traces of acetic anhydride were removed by adding a little alcohol and evaporating again and finally heating it 120°C . The yield agreed with that calculated for a diacetyl derivative. This substance was readily soluble in hot water and when the solution was cooled slowly a mixture of needles and rectangular prisms separated. The diacetyl compound was converted to a purine as follows.

Five grams of the acetylated 2-oxo-4-methyl-5,6-diaminopyrimidine were dissolved in 50 cc of water containing 2.5 grams of potassium hydroxide and the solution was evaporated to dryness. The resulting potassium salt was then heated in an oil bath at $220^{\circ}\text{--}240^{\circ}\text{C}$ until water was no longer evolved. The reaction product, which was apparently a potassium salt, was dissolved in hot water, the solution was filtered to remove a slight turbidity and was afterwards acidified with acetic acid. A crystalline precipitate formed rapidly. This was purified by dissolving it in dilute ammonia, clarifying with blood coal, boiling off excess of ammonia and precipitating with acetic acid. The crystals were small prisms with square ends. They did not melt at 315°C although they turned brown slowly at that temperature. They were difficultly soluble in hot water, very slightly soluble in hot alcohol and insoluble in boiling benzene. They gave a murexide reaction. The yield of this purine was 70 per cent, calculated from the weight of 2-oxo-4-methyl-5,6-diaminopyrimidine.

0.2009 gram of substance gave 0.3758 gram of CO_2 and 0.0880 gram of H_2O .

	Calculated for $\text{C}_7\text{H}_6\text{N}_4\text{O}_2$	Found
C	51.21	51.02
H	4.87	4.86
N	34.14	34.26

THE CREATINE CONTENT OF MUSCLE UNDER NORMAL CONDITIONS ITS RELATION TO THE URINARY CREATININE¹

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Although much attention has been devoted to urinary creatinine since the introduction of Folin's simple colorimetric method for its estimation in 1901, relatively little consideration has been given to the supposedly related muscle creatine. It is reasonable to believe that a knowledge of the creatine content of muscle, under normal and likewise abnormal conditions, may greatly aid in elucidating the various phases of creatine-creatinine metabolism. Attention has recently been called to some of these points by Mendel and Rose² who have cited the more important researches upon muscle creatine. van Hoogenhuyze and Verploegh³ were the first workers to employ the colorimetric method for the estimation of creatine in muscle, but to Mellanby⁴ is due the credit of first giving the subject any considerable attention. In addition, a few analyses have been reported by Dorner,⁵ Emmett and Grindley,⁶ Saiki,⁷ Graham-Brown and Catheart,⁸ von Furth and

¹ A preliminary report of this work was presented to the Society for Experimental Biology and Medicine October 16, 1912 see *Proceedings*, x, p 10, 1912

² Mendel and Rose *this Journal*, x, p 255, 1911

³ van Hoogenhuyze and Verploegh *Zeitschr f physiol Chem*, xlv, p 433, 1905

⁴ Mellanby *Journ of Physiol*, xxxvi, p 447, 1908

⁵ Dorner *Zeitschr f physiol Chem*, li, p 259, 1907

⁶ Emmett and Grindley *this Journal*, iii, p 499, 1907

⁷ Saiki *ibid*, iv, p 487, 1908

⁸ Graham-Brown and Catheart *Biochem Journ*, iv, p 423, 1909

In the instances in which these figures were given in terms of creatinine, they have been converted to creatine. With the exception of the results of Graham-Brown and Cathcart, the results are comparatively uniform. In their experiments, it should perhaps be noted that the figures were obtained with comparatively small samples of muscle (taken from one leg previous to the stimulation of the muscle on the opposite side).

Chisolm's results for the creatine content of the moist muscle of six healthy adults are as follows: 0.257, 0.290, 0.271, 0.280, 0.251 and 0.268 per cent, an average of 0.270.

Below is further appended the creatine content of muscle in a variety of animals as determined by various investigators with the colorimetric method, though in single experiments in many instances.

Reported figures (averages) for muscle creatine in man and various animals

ANIMAL	CREATINE IN MUSCLE	OBSERVER	ANIMAL	CREATINE IN MUSCLE	OBSERVER*
	per cent			per cent	
Rabbit	0.51	Dorner	Horse	0.35	von Fürth and Schwarz
	0.51	Mellanby			
	0.41	Graham- Brown and	Dog	0.31	von Fürth and Schwarz
		Cathcart			
Pig	0.50	Mendel and Rose	Man	0.27	Chisolm
	0.45	van Hoogen- huyze and	Guinea pig	0.37	Mellanby
		Verploegh	Rat	0.35	Mellanby
	0.38	Mellanby	Hen	0.36	Mellanby
Ox	0.44	van Hoogen- huyze and		0.41	Mendel and Rose
		Verploegh	Frog	0.30	Mellanby
	0.44	Emmett and Grindley	Cod	0.35	Mellanby
	0.38	Mellanby	Lampfrey	0.29	Mellanby
Sheep	0.41	van Hoogen- huyze and	Skate	0.28	Mellanby
		Verploegh	Bonito	0.65	Okuda
	0.38	van Hoogen- huyze and	Tunny fish	0.50	Okuda
		Verploegh	Salmon	0.56	Okuda
Horse	0.38	van Hoogen- huyze and	Snapper	0.75	Okuda
		Verploegh	Carp	0.42	Okuda
			Shark	0.66	Okuda

* References previously cited

to fourteen days until it was believed that the correct daily creatinine elimination had been ascertained. Determinations were also made for creatine. At the end of the period, the animals were killed, body weights were taken daily previous to feeding, though the weights recorded in the table below were those taken just prior to the death of the animals.

Our results upon the creatine concentration of fresh muscle will be considered first.

Creatine content of rabbit muscle

Table I gives the results which we have obtained from a series of twenty presumably normal rabbits.

TABLE I
Creatine content of rabbit muscle

ANIMAL NUMBER	BODY WEIGHT	WEIGHT OF MUSCLE SAMPLE	CREATINE IN FRESH MUSCLE
	<i>kgs</i>	<i>grams</i>	<i>per cent</i>
1	1 48	100	0 522
2	1 55	100	0 540
3	1 49	100	0 522
4	1 77	100	0 522
5	1 39	100	0 522
6	2 12	100	0 494
9	2 05	100	0 497
10	2 13	{ 100	{ 0 524
		{ 100	{ 0 522
11	1 87	100	0 522
12	2 56	100	0 495
15	1 99	100	0 522
25	1 74	{ 75	{ 0 522
		{ 85	{ 0 527
27	1 35	100	0 517
28	1 90	100	0 522
29	1 85	100	0 521
30	1 60	100	0 534
31	1 80	100	0 522
32	1 66	100	0 495
33	2 05	100	0 522
34	1 98	100	0 516
Average content of creatine			0 518

This series includes the animals made the subjects of our creatine-creatinine metabolism study and, in addition, a number of miscellaneous normal rabbits from which only muscle samples were obtained. In the first half dozen determinations, the colorimetric readings were first recorded without calculating our results. When this was done, we were surprised at the unusually uniform results. Our subsequent experiments entirely confirmed this. In fact, in going back over our notes of the various observations, incidents had been recorded which would possibly account for the slightly lower values in two cases. In animal 9, a correction of 5 mgms had to be added for creatine found in the seventh extract, while an extract of the coagulated protein contained 12 mgms, indicating incomplete extraction. With animal 32, note was made that considerable difficulty was experienced in obtaining a good coagulation of the protein, though this fact was not recalled at the time the colorimetric estimation was made. Animal 12 was found to have a subcutaneous abscess. Even if we include these results, the creatine content of fresh rabbit muscle is found to be very definite and, as will appear below, characteristic of the animal.

In animal 10, the creatine contents of the left and right hind legs were found to be practically identical. In animal 25, the creatine content of the muscle of the right leg was found to be 0.522 per cent, that of the muscle of the back, adjacent to the vertebral column, 0.527 per cent. After allowing the left leg to remain at room temperature (very warm, July 20) for twenty-four hours, a creatine estimation of the muscle showed 0.482 per cent.

Creatine content of dog muscle

In Table II are recorded five experiments on the creatine content of dog muscle. These figures are likewise very uniform, but considerably lower than in the case of rabbit muscle.

TABLE II
Creatine content of dog muscle

ANIMAL NUMBER	ESTIMATED WEIGHT	WEIGHT OF MUSCLE SAMPLE	CREATINE IN FRESH MUSCLE
	<i>kgs</i>	<i>grams</i>	<i>per cent</i>
1	18	100	0 370
2	6	75	0 364
3	6	100	0 361
4	6	100	0 367
5	12	90	0 373
Average content of creatine			0 367

Creatine content of cat muscle

The figures for the creatine content of cat muscle, as shown in Table III, are not as uniform as found in the case of the rabbit and dog, though, with two or three exceptions, the figures are fairly constant. Cat 3 was a starving animal, whose original weight was 3 19 kgs, and had been deprived of food for about forty days. Cat 11 was noted to be very poorly nourished. Cat 9 was a moderately fat pregnant animal. The low figure for Cat 16 cannot be accounted for. Although the variations are possibly connected with the nutritional state of the animal (fat or lean—ingestion of creatine with meat) we have no data to ex-

TABLE III
Creatine content of cat muscle

ANIMAL NUMBER	BODY WEIGHT	WEIGHT OF MUSCLE SAMPLE	CREATINE IN FRESH MUSCLE
	<i>kgs</i>	<i>grams</i>	<i>per cent</i>
11	0 77	50	0 515
15	0 88	58	0 438
14	0 92	40	0 421
13	1 00	50	0 463
16	1 26	77	0 383
12	1 28	61	0 440
3	2 02	100	0 470
2	2 07	100	0 420
5	2 26	100	0 443
9	2 84	100	0 494
Average content of creatine			0 449

plain these variations. The weight does not appear to influence the content of creatine in the adult.

Influence of growth upon the creatine content of cat muscle

In Table IV, figures are recorded for the muscle creatine of kittens from the same litter, but at different ages. These figures are in accord with the observations of Mellinby¹ on rabbits and chickens of various ages.

TABLE IV

Effect of growth on the creatine content of cat muscle

ANIMAL NUMBER	WEIGHT OF ANIMAL grams	AGE weeks	CREATINE IN FRESH MUSCLE
			per cent
4	215	2	0.224
6	300	3	0.285
7	386	5	0.309
8	464	6	0.341
10	670	7	0.467

Creatine content of human muscle

In two instances we have been able to obtain satisfactory samples of human muscle at autopsy with the following results.

The objection may be raised that the figures given for the muscle creatine of different animals are not comparable, because of the possibility of differences in composition, such as content of moisture or rather of solid matter. Estimation of moisture in supposedly

TABLE V

Creatine content of human muscle

SEX	CAUSE OF DEATH	SOURCE OF MUSCLE	CREATINE IN FRESH MUSCLE	MOISTURE OF MUSCLE	NITROGEN OF MUSCLE
			per cent	per cent	per cent
F	Peritonitis	Abdominal	0.396	76.4	3.67
M	Amputation for sarcoma of the leg	Leg	0.391	76.3	3.44

¹ *Loc. cit.* p. 473

normal samples of muscle has been made in only a few instances, because the figures were always found to be very uniform, not only for the same animal, but for animals of different species. The following figures for moisture, in addition to those for the human muscle above, may be cited: Dog 1, 75.5 per cent, Cat 5, 74.8 per cent, Cat 11, 75.8 per cent, Rabbit 12, 75.6 per cent, Rabbit 25, 74.5 per cent, Rabbit 35, 75.0 per cent.

Creatinine coefficient and muscle creatine

It is a curious fact that the creatinine coefficient (milligrams of creatinine nitrogen eliminated per kilogram of body weight) of the rabbit is fully one-third higher than that found in man and the various experimental animals, dog, pig,¹⁶ cow¹⁷ and guinea pig.¹⁸ In forty rabbits in which we have had occasion to accurately determine the creatinine-nitrogen coefficient, it has been found to average 14.3. When the creatinine coefficients of the rabbit, dog and man are compared with the percentage content of muscle creatine in the same species, as shown in Table VI, an interesting and, as we believe, more than an accidental relationship is revealed. If creatinine has its origin in muscle creatine, or some common precursor substance, we would naturally expect from the creatinine coefficient of the rabbit to find that the muscle of this animal had a relatively high percentage content of creatine.

Although our figures given in Table VI for human muscle only represent two observations and are considerably higher than those given by Chisolm, we are not inclined to accept the figures of Chisolm as the normal, for even in children and in certain pathological conditions,¹⁹ we have failed to obtain results as low as those

¹⁶ Figures calculated from data of McCollum. *Amer. Journ. of Physiol.*, xix, p. 210, 1911.

¹⁷ Figures calculated from data reported by Hart, McCollum, Steenbock and Humphrey. The University of Wisconsin Agricultural Experiment Station, Research Bulletin 17, June 1911.

¹⁸ Myers and Fine. Unpublished data.

¹⁹ In a baby 3 months old, weighing 3.2 kgms., and dying from cerebrospinal meningitis, muscle from the back and groin contained 0.321 per cent creatine. Muscle taken from the thigh in a case of marked edema in a man, gave 0.311 per cent of creatine and 80.5 per cent moisture. Muscle taken from the abdominal wall in a woman dying from general peritonitis

TABLE VI

Relation between muscle creatine and creatinine coefficient

ANIMAL	NUMBER OF EXPERIMENTS	CONTENT OF CREATINE IN MUSCLE	NUMBER OF EXPERIMENTS	CREATININE COEFFICIENT
		per cent		
Rabbit	20 normal animals	0.52	10 normal animals	14.3
Man	2 experiments	0.39	3 normal men about 27 years of age	9.0
Dog	5 normal animals	0.37	3 normal animals	8.4

reported by this author for supposedly normal muscle. We hope to further extend our studies on human muscle. According to Shaffer²⁰ the normal creatinine coefficient in man falls between 7 and 11. In three normal well-developed men about twenty-seven years of age figures of 8.8, 9.0 and 9.2 respectively were obtained.²¹

The average of the figures which we have tabulated above for the creatine content of dog muscle probably represents the correct value, judging from the uniformity of the results upon which this figure was based. The creatinine coefficient in the dog shows some little variation, though the figure tabulated above probably approximates the average result. Shaffer²² states in this connection, "The kreatinin coefficients of normal dogs are practically the same as those given above for man. Wolf and Osterberg found an average of 8.2 for one dog and 7.0 for another. Results of my own from four dogs lie between the above figures."

contained 0.322 per cent creatine and had a moisture content of 80.2 per cent. The differences in the moisture content of the muscle in the last two instances, as compared with the figures tabulated above, practically accounts for the difference in the creatine results, *i. e.*, by reducing these last results to the tabulated water content, figures of about 0.384 are obtained.

²⁰ Shaffer *Amer Journ of Physiol*, xviii, p 1, 1908

²¹ Figures calculated from experiments on W. W. H., V. C. M. and J. F. L. reported by Mendel and Myers *ibid*, xxvi, p 77, 1910, and Mendel and Hilditch *ibid*, xxvii, p 1, 1910

²² *Loc cit*, p 5

The slight difference in the creatine content of dog muscle and human muscle, and in the creatinine coefficients of the same species (this statement could probably be made with regard to the pig, ox and guinea pig) is very interesting when compared with the uniformly greater values for the muscle creatine and the creatinine coefficient in the rabbit. The rabbit appears to be a unique animal in this respect. The comparatively high creatinine coefficient and high muscle creatine of the rabbit is very suggestive of the origin of creatinine from creatine.

With the view of ascertaining further data bearing on this point, experiments were planned in an endeavor to determine whether in a given animal, such as the rabbit, there was a constant relationship between the total creatine of the body and the daily creatinine. This ratio was ascertained in a series of one growing and eleven adult rabbits. The protocols follow.

Rabbit 1, a gray-brown male animal, was placed under observation on January 4, 1911, after a preliminary carrot diet period. The creatinine elimination was determined on five successive days as 19.6, 19.8, 19.4, 20.0 and 20.8 mgms creatinine N, an average of 19.9 mgms, giving a coefficient of 13.4. Creatine was absent from the urine. On the morning of January 11, the animal weighed 1.48 kgms, previous to eating and at 10 a.m. was killed by bleeding after ether anesthesia. The total skinned and eviscerated carcass plus heart weighed 804 grams. The viscera minus heart weighed 392 grams. The carcass contained 2.633 grams creatine and the viscera, 0.082 gram.

Rabbit 2, a brown-gray male rabbit, after the usual preliminary uniform carrot diet, was placed under observation on January 4, and the creatinine elimination determined on seven successive days to be 23.5, 22.6, 20.3, 27.0, 21.5, 23.5 and 21.2 mgms creatinine N, an average of 22.8 mgms. This figure gave a coefficient of 14.7 calculated from its body weight of 1.55 kgms, a weight which the animal maintained throughout the experiment. The rabbit eliminated no creatine. When killed on January 15, the skinned and eviscerated carcass plus the heart weighed 830 grams and contained 3.013 grams creatine. The viscera minus the heart weighed 460 grams and contained 0.069 gram creatine.

Rabbit 3, a female albino rabbit, after the customary preliminary diet was placed under study on January 9. The seven daily creatinine nitrogens were 24.7, 22.6, 23.4, 22.3, 19.9, 21.2 and 22.3 mgms, with an average of 22.3, giving a creatinine coefficient of 15.0 with the body weight of 1.49 kgms obtained on the morning of January 18, without food previous to being killed. No creatine was eliminated in the urine. The skinned and eviscerated carcass plus heart weighed 878 grams and contained 3.069 grams of creatine.

Rabbit 4, a female albino, was placed under observation in the usual way on January 9. The creatinine N elimination for five days was 21.1, 23.5, 24.7, 23.1 and 22.3 mgms, an average of 23.6 mgms, giving a creatinine coefficient of 13.4 with the body weight of 1.77 kgms. The urine contained no creatine. The animal was killed on January 23. The skinned and eviscerated carcass plus the heart weighed 940 grams and contained 3.096 grams of creatine.

Rabbit 5, a black male, was studied in the usual way from February 1-6. The creatinine N elimination per day for five days was 20.1, 18.1, 19.4, 20.5 and 19.8 mgms, an average of 19.6 mgms. The body weight was 1.39 kgms, giving a coefficient of 11.1. The animal eliminated no creatine. The skinned and eviscerated carcass plus the heart weighed 790 grams and contained 2.514 grams creatine.

Rabbit 6, a female albino of 2.12 kgms weight, eliminated from January 9-14, daily, 32.2, 29.1, 29.1, 27.0 and 28.7 mgms creatinine N, averaging 29.2 mgms. The creatinine coefficient was 13.7, no creatinine was eliminated. The animal was killed on January 29, the skinned and eviscerated carcass plus the heart weighing 1172 grams. It contained 3.535 grams creatine.

Rabbit 7, was a growing female albino, which showed a gain in weight from 0.59 to 0.69 kgm during two weeks of observation, after beginning the experiment on February 24. The daily creatinine N elimination for 11 days was 6.9, 7.0, 6.5, 7.1, 7.3, 7.3, 7.1, 7.1, 8.0, 7.2 and 7.2, averaging 7.2 mgms, and giving a coefficient of 10.4 with the body weight of 0.69 kgms. There was a daily elimination of between 1 and 2 mgms of creatine N.²³ The weight of the skinned, eviscerated carcass employed for analysis was 280 grams and this contained 0.917 gram of creatine.

Rabbit 9, a light-brown female adult animal, weighing 2.05 kgms, eliminated daily the following amounts of creatinine N: 30.1, 30.6, 31.7, 29.1, 25.1, 25.1, 27.8, 25.1, 28.7, 27.6, 28.7, 27.0, 27.4, 27.4, 28.7, 28.7, 28.7, 29.3, 29.1 and 29.6 mgms, an average of 28.3 mgms, giving a coefficient of 13.8. Traces of creatine were detected in the urine on several days. The animal was killed on March 4 and the skinned and eviscerated carcass plus heart weighed 1140 grams and contained 3.385 grams of creatine.

Rabbit 10, a dark-gray female, weighing 2.13 kgms, eliminated daily 32.8, 32.9, 32.4, 31.5, 39.2, 32.4, 31.9 and 32.1 mgms of creatinine N, averaging 32.3 mgms. This gave a coefficient of 15.2. The urine contained no creatine. The animal was killed on February 21 and the skinned and eviscerated carcass plus the heart weighed 1185 grams and contained 3.899 grams of creatine.

Rabbit 11, an albino female, after the usual preliminary diet was found to eliminate 27.0, 25.8, 25.8, 24.7, 26.6, 25.8, 25.1, 23.5, 24.5, 24.8, 26.0 and

²³ The urine of growing animals contains creatine for some as yet unexplained reason. Cf. Closson *Amer Journ of Physiol*, *xvi*, p. 252, 1906; Rose *this Journal*, *x*, p. 265, 1911; Folin and Denis *ibid*, *xi*, p. 253, 1912. We have also been able to verify this observation on children in some unpublished results.

24.8 mgms of creatinine N per day, averaging 25.4 mgms and giving a coefficient of 13.5 with its body weight of 1.87 kgms. No creatine was eliminated in the urine. The animal was killed on February 28, the skinned, eviscerated carcass weighed 965 grams and contained 3.070 grams creatine.

Rabbit 12, a gray female, was placed under observation in the usual way on March 18. The daily creatinine N elimination until March 29 was 37.9, 39.0, 35.1, 36.3, 30.1, 38.3, 38.3, 38.3, 37.4, 32.9 and 34.0 mgms. On April 9 this animal gave birth to three young. From April 11 to 16 the daily creatinine N was 38.3, 38.3, 40.5, 37.6 and 40.5 mgms, an average of 37.1 mgms for both periods. This gave a creatinine coefficient of 14.5 with the body weight of 2.56 kgms. No creatine was detected in the urine. On April 16 the animal was killed. A subcutaneous abscess, weighing about 50 grams, was found in the skin on the ventral surface of the body. The skinned, eviscerated carcass weighed 1480 grams and contained a moderate amount of fat, which was removed prior to extraction. The total amount of creatine was 4.317 grams.

Rabbit 15 was an albino female. Eight embryos were found when the animal was killed on March 23. The daily creatinine N elimination was 27.4, 27.0, 31.0, 28.5 and 27.0 mgms, averaging 28.2 mgms. This gave a coefficient of 15.2 when computed from the body weight of 1.89 kgms. The urine contained no creatine. The weight of the skinned, eviscerated carcass was 1035 grams and this contained 3.370 grams of creatine.

In the above protocols, the samples of muscle taken for creatine analysis have not been considered, as they do not appear to be concerned in the problem at hand, these special data having been recorded in Table I. Any creatine present in the internal organs or blood has not been tabulated, because it was thought that these small amounts, probably not over 0.1 gram in any case, would not invalidate our deductions.

In Table VII are given our results for this series of experiments. An inspection of this table shows that in the first five animals of nearly the same weight, about 2 kgms (exclusive of the first rabbit, No. 12, weighing 2.56), the ratio between the total body creatine and the daily creatinine, was very constant, averaging 44.7:1. This included animals with high creatinine coefficients (15.3) and low coefficients (13.5). In other words, animals with a high creatinine elimination had a proportionately high body content of creatine. In rabbit 12, weighing 2.56 kgms, the creatine-creatinine ratio was 43.2:1. With smaller animals, the ratio of the creatine to the creatinine was found increased, e.g., in rabbit 5, weighing 1.39 kgms, it was observed to be 53.3:1. In the growing rabbit 7, which had a low creatinine coefficient, was eliminat-

TABLE VII

Relation between total creatine of tissues and creatinine of urine in the rabbit Data tabulated in order of body weight

NUMBER OF MIL	BODY WEIGHT AT DEATH	WEIGHT OF CAR C %	TOTAL BODY CREATINE	AVERAGE DAILY URINARY CREATININE	RATIO OF BODY CREATINE TO DAILY CREATININE	AVERAGE DAILY CREATININE EXCRETION	CREATININE CO- EFFICIENT	CONTENT OF BODY CREATINE
	kgms	grams	grams	mgms		mgms	mgms	per cent
12	2.56	1180	1.317	99.9	13.2	37.1	11.5	0.169
10	2.13	1185	3.899	86.9	11.9	32.3	15.2	0.183
6	2.12	1172	3.535	78.6	15.0	29.2	13.7	0.167
9	2.05	1140	3.385	76.2	14.4	28.3	13.8	0.165
15	1.89	1035	3.370	75.9	14.1	28.2	15.3	0.178
11	1.87	965	3.070	68.4	14.9	25.1	13.5	0.164
4	1.77	910	3.096	63.5	18.8	23.6	13.1	0.176
2	1.55	830	3.013	61.1	19.1	22.8	14.7	0.195
3	1.49	878	3.069	60.0	51.1	22.3	15.0	0.206
1	1.48	801	2.633	53.5	49.2	19.9	13.4	0.178
5	1.39	790	2.811	52.8	53.3	19.6	14.1	0.202
7	0.69	280	0.917	19.2	19.3	7.1	10.4	0.138

ing creatine and had a low percentage of body creatine, the ratio of total creatine to creatinine was 49.3 : 1, a figure not essentially different from the other rabbits next in size. It is possible that the slightly high ratios observed in the last six animals may all be explained on the basis of age. We appreciate the desirability of further data on this point. The ratio between the total body creatine and daily creatinine would appear to be the most reliable means of comparing these two supposedly related substances, because the factor of body weight is here eliminated from the calculations.

Nevertheless in Table VIII, the data from ten rabbits, exclusive of Nos. 12 and 7, the two extremes, have been arranged in order of the creatinine coefficients. The animals with a low percentage content of body creatine are seen to have low creatinine coefficients and *vice versa*. It is not believed that these figures are simply referable to variations in body weight which enter into these calculations. We endeavored to make our body weight figures as reliable as possible, the weight being taken in the morning prior to giving food and after compression of the bladder.

TABLE VIII

Relation between per cent of body creatine and creatinine coefficient Data tabulated in order of creatinine coefficients

NUMBER OF ANIMAL	BODY WEIGHT	CREATININE COEFFICIENT	CONTENT OF CREATINE IN BODY	NUMBER OF ANIMAL	BODY WEIGHT	CREATININE COEFFICIENT	CONTENT OF CREATINE IN BODY
	kgms		per cent		kgms		per cent
1	1.77	13.1	0.176	5	1.39	14.1	0.202
1	1.48	13.4	0.178	2	1.55	14.7	0.195
11	1.87	13.5	0.164	3	1.49	15.0	0.206
6	2.12	13.7	0.167	10	2.13	15.2	0.183
9	2.05	13.8	0.165	15	1.89	15.3	0.178
Averages		13.6	0.170	Averages		15.0	0.193

In this way a period of twelve to fourteen hours generally elapsed after the taking of food. An inspection of Table VII shows that the creatinine elimination follows in every case the figures for body weight, which is an indication of the reliability of these figures.

This uniform relation between the creatinine elimination and the body weight, falls in line with the original statement of Folin¹ that "The chief factor determining the amount of creatinine eliminated appears to be the weight of the person," a point subsequently discussed by Benedict and one of us,²⁵ and by Shaffer.²⁶ The reason in this case is, in our opinion, clear. The relative proportion of the body tissues in a series of rabbits will be found relatively constant, *i.e.*, the adipose tissue is always small, in consequence of which the muscular tissue assumes a very uniform relation to the rest of the body. To this uniform proportion of muscle with its constant content of creatine, we are inclined to ascribe the constant relation between body weight and urinary creatinine. Shaffer's statement that "Creatinin¹⁵ is an index of some special process of normal metabolism taking place largely, if not wholly, in the muscles," appears in a new and added light.

¹ Folin *Amer Journ of Physiol*, viii, p. 85, 1905

²⁵ Benedict and Myers *ibid*, xviii, p. 392, 1907

²⁶ Shaffer *ibid*, xviii, p. 4, 1908

Bearing in mind the constancy in the content of creatine in the striated muscle of the rabbit, it would appear possible, from our figures for total body creatine or per cent of body creatine (Table VII), to calculate the total amount of striated muscle in the body of the rabbit and its relative proportion to the body weight. Taking the average figure for the content of body creatine, about 0.182 per cent, 35 per cent of the weight of the rabbit would be in the form of muscle tissue. Should it be found possible to ascertain the factor influencing the relation between the total body creatine and the urinary creatinine, the amount of muscle tissue could be calculated from the daily creatinine elimination.

In a series of experiments (18) upon starving rabbits, which will be considered in a subsequent paper and have already been reported in abstract,⁷ it has been found that the creatine appearing in the urine in this condition accounts very largely for the creatine which has been lost from the muscle tissue. In a short fast this urinary creatine almost entirely accounts for the creatine lost by the tissues, but with an increase in the length of the fast this is progressively less true. This deficit we are inclined to ascribe to the creatinine eliminated during the period of starvation. Should this be the case, it would greatly strengthen the idea of the metabolic origin of creatinine from creatine or some common precursor substance. There are many factors which need to be considered in this connection such as specific enzymes, the oxidative power of the body toward these two compounds, etc. Experiments are in progress to determine the fate of administered creatine and creatinine, with methods similar to those outlined above.

CONCLUSIONS

It has been pointed out that *the creatine content of muscle* in two typical animals, viz., the rabbit (herbivorous) and the dog (carnivorous) *is very constant*. For the cat the figures were somewhat less constant. Not only is the content of muscle creatine relatively constant for a given animal, but the figures appear to be *distinctive*, 0.52 per cent for the rabbit, 0.45 per cent for the cat, 0.37 per cent for the dog, 0.39 per cent for man, etc.

⁷ Myers and Fine *Proc Soc for Exp Biol and Med* x, p 12 1912

Another fact to which we have called attention and which we believe to be of particular interest in this connection, is the creatinine coefficients of the same animals. *The creatinine elimination appears to bear a distinct relation to the percentage content of muscle creatine in a given species.* The creatinine coefficient of the rabbit averages fully a third more than that of the dog and man, and we find similar relations with regard to the muscle creatine.

The constancy in the content of muscle creatine offers a satisfactory explanation for the constancy in the daily elimination of creatinine, first noted by Folin and subsequently confirmed by many workers. We have repeatedly observed that the daily creatinine in the rabbit may show almost identical figures for periods of days.

In rabbits of a nearly uniform weight it has been shown that *a constant relation exists between the total creatine of the body and the daily creatinine elimination.* Animals with a high creatinine coefficient appear to have a proportionately higher content of body creatine.

In our series of twelve rabbits, *the figures for the urinary creatinine follow the figures for body weight in every case.* The reason for this is, in our opinion, the uniform muscular development of the rabbit—very little adipose tissue—resulting in a relatively uniform body content of creatine, *i e*, of creatinine-forming substance.

Based upon our figures for the creatine content of rabbit muscle and of the creatine concentration of the body, we have calculated that *striated muscle forms 35 per cent of the body weight in this animal.*

A NEW METHOD FOR DRYING TISSUES AND FLUIDS

By JACOB ROSENBLOOM

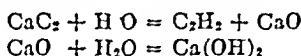
(From the Laboratory of Biochemistry of the University of Pittsburgh,
Pittsburgh, Pa)

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The methods in use at present for drying tissues and fluids may be classified as follows (1) Drying by means of salts, anhydrous sodium or calcium sulphate, (2) drying by means of alcohol, (3) drying in air or neutral gas

As is well known all of these methods have many disadvantages. The methods based on drying by means of anhydrous salts often cannot be used because the mass obtained is inconveniently large when one expects to carry out a Soxhlet extraction on the material.

The method to be described is based on the reaction between calcium carbide and water



and has many advantages, especially when drying tissues preliminary to the extraction of the contained lipins.

It may be noted that most of the water undergoes reaction with the carbide to form acetylene, the mass of material being much less on that account than when using calcium sulphate or sodium sulphate, because the water is taken up by these salts to form the hydrated salt and the water still remains in the material. Also the calcium oxide formed in the reaction is a dehydrating agent and facilitates the drying.¹ Another advantage in the calcium carbide method is that on account of the hardness of the carbide it is unnecessary to add sand in the mixing and grinding of the material.

¹ The presence of calcium oxide is especially advantageous when drying bile before extracting lipins as the calcium oxide forms an insoluble compound with the bile pigments and prevents their extraction by the solvents used.

Also after the substance is dried, on treatment with any of the lipin solvents it takes on a granular form, thereby giving the solvent the best chance of reaching the soluble lipins enclosed in the large amount of insoluble substance making up the tissue

This method may be used without any precaution at all when one simply wants to extract *qualitatively* substances from tissues or fluids or for class-room work, such as the preparation of cholesterol from brain. However, when using it for drying material before using the material for *quantitative* analysis, I have so far only found one disadvantage. Owing to the fact that the reaction between calcium carbide and water is markedly exothermic, it is possible that the heat developed might decompose certain of the constituents. This can be minimized by adding the carbide in small amounts to the material to be dried, contained in a porcelain dish packed in ice.

When using the carbide for drying fluids, one simply adds the carbide in lumps until the material has lost its liquid form, then the powdered carbide is added till the drying is complete. The same method is used for tissues, with the addition of thoroughly mixing the carbide by grinding just as in the other methods.

On account of the fact that acetylene is explosive and poisonous it is wise to carry out the drying under a hood, free from flames.

In regard to any possible change that the acetylene might produce in the chemical nature of the constituents of the tissues or fluids dried by this method I can say that no differences could be detected in the character of the lipins of heart muscle (ox) and of urine, when obtained from material dried by this method, when compared with that obtained from heart muscle and urine, dried by means of anhydrous calcium sulphate.

PROTEIN METABOLISM FROM THE STANDPOINT OF BLOOD AND TISSUE ANALYSIS

SIXTH PAPER

ON URIC ACID, UREA AND TOTAL NON-PROTEIN NITROGEN IN HUMAN BLOOD

By OTTO FOLIN AND W. DENIS

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

(Received for publication, December 31, 1912.)

In earlier papers of this series we have reported studies on the non-protein nitrogenous products of the blood from the standpoint of absorption. The definite nature of the results recorded was largely due to the fact that the new technique employed proved suitable for the work. In the absence of unusually rapid absorption or of abnormally high levels of protein destruction (as in fevers) the non-protein nitrogen of the blood must be a more or less sharp index to the efficiency of the kidneys in removing the waste nitrogenous products circulating in the blood. The investigation of the non-protein nitrogenous constituents of human blood is especially interesting from this point of view because of its possible clinical bearings.

We are well aware, of course, that a great many investigations have already been made in this field, in fact, ever since the time of Bright, clinicians have been interested in the "retention nitrogen" of the blood and have tried hard to connect it in one way or another with "uremia".¹ But on the whole the results have not proved particularly convincing, instructive or helpful, either to physiologists or to clinicians. By means of our new methods, however, we expected at least to be able to measure various degrees of nitrogen retention and urea accumulation due to kidney inefficiency with much greater accuracy than had heretofore been possible.

¹ For a résumé of much of the literature on this subject see Obermayer and Popper *Zeitschr f klin Med*, pp 72, 332, 1911.

The ammonia in blood has received a large amount of attention in the study of nephritis. The determination of this constituent in blood is, however, far more difficult than any of the earlier investigators realized, and their results are probably worthless. The amount in normal blood is, as we now know, so minute as to be entirely out of reach by titration methods.² We have not yet made any investigations as to the extent to which it may be found in pathological blood.

So far as the uric acid in human blood is concerned we have heretofore had to remain satisfied with the theoretical deduction that it must always be there because it is rather abundant in the urine and because its presence in the blood in cases of gout, leukemia and lead poisoning has been demonstrated by a number of different investigators. To be sure, Garrod claimed long ago to have demonstrated the presence of uric acid in normal human blood as well as in the blood of those suffering from gout or nephritis.³ But there is room for the suspicion that Garrod drew to a considerable extent on his imagination when applying the "microscopic identification," the "thread test" and the murexide reaction for the demonstration of uric acid in blood. The modern precipitation methods (the methods of Salkowski and of Krüger) have proved inadequate for the task of finding uric acid in normal human blood. Magnus-Levy, Klemperer, von Jaksch, Brugsch and Schittenhelm, all have failed to find the uric acid in normal blood by the help of these methods. A few, notably Petré, have obtained positive results. But at all events the detection of uric acid in normal human blood has been extremely uncertain, if not entirely impossible, and a "positive uric acid test" is accordingly extensively used and generally accepted as a valuable aid in the diagnosis of gout.⁴

As already indicated in our paper on a method for determining uric acid in blood⁵ there is as a matter of fact enough uric acid in normal human blood to make its quantitative determination (by that method) almost as simple as the determination of the urea or the total non-protein nitrogen.

² This *Journal*, vi, p. 167, 1912.

³ *The Nature and Treatment of Gout*.

⁴ See Brugsch and Schittenhelm *Zeitschr. f. exp. Path. u. Ther.*, iv, pp. 438, 446, 480, 532, 538, 551, 1907. See also Gudzent *Deutsch. med. Wochenschr.*, xxxviii, p. 603, 1912.

⁵ See the last number of this *Journal*, p. 469.

All three determinations can be made without requiring more than 20 to 30 cubic centimeters of blood. We have therefore in a combination of these three determinations practically a new system of blood analysis—certainly one well worth trying out on human blood for clinical purposes.

As a preliminary to such work and having for the first time a method capable of detecting extremely minute amounts of uric acid we thought it worth while also to make a general survey of the blood of different kinds of animals. The results of this survey are given in table 1.

TABLE 1

Uric acid, total non-protein nitrogen and urea nitrogen in blood
(The figures represent milligrams per 100 grams of blood.)

	URIC ACID	NON PROTEIN NITROGEN	UREA NITROGEN
Rabbit (6 cases)	0.05	31	13
Sheep (mixed blood)	0.05	28	13
Pig (mixed blood)	0.05	32	14
Horse (1 case, antitoxin animal)	0.05	54	28
Monkey (1 case polyomyelitis)	0.05	60	38
Beef (mixed blood)	0.2	24	14
Cat (2 cases diet, liver)	0.2	60	34
Cat (2 cases diet, milk and eggs)	0.2	67	37
Cat (2 cases diet, rice and cream)	0.2	31	20
Chicken (6 cases, mixed blood)	4.9	32	8
Duck (4 cases, mixed blood)	4.8	34	7
Goose (1 case)	4.8	26	8

From the results obtained it would appear that the uric acid in the blood of rabbit, sheep and horse is almost nil, and that in the case of the other animals the amount though a trifle larger is still extraordinarily small—0.2 of a milligram or less per 100 grams of blood. In bird's blood where the origin of the uric acid is entirely different from that of mammals the amount is, of course, very much larger, but still is not as large as we had expected to find it. It might not be out of place in this connection to call attention to the small amounts of urea found in the blood of birds as compared with the amounts occurring in blood of mammals.

The results of our first survey of the uric acid in human blood are given in table 2. The figures can scarcely be said to represent the strictly normal variations for no attempt was made to select physically normal persons. All the samples of blood for the analyses recorded in this table were obtained from newly admitted patients at the Massachusetts Psychopathic Hospital in Boston.*

The uric acid figures of table 2 are arranged according to the amount of uric acid found. We have also made an arbitrary division of the figures into three groups. We have done this only to facilitate inspection with regard to the amount of uric acid most frequently found in human blood. The uric acid figures

TABLE 2

Uric acid in unselected human blood

(The figures represent milligrams per 100 grams of blood.)

Group 1, 5 cases	{	0.7 0.9	0.8	0.8	0.9
	{	1.0 1.2 1.4 1.5 1.6 2.0	1.0 1.3 1.5 1.6 1.6 2.0	1.2 1.4 1.5 1.6 1.6 2.0	1.2 1.4 1.5 1.6 1.8 2.0
Group 2, 22 cases	{	2.2 2.8 3.0	2.4 2.8 3.5	2.5 2.8 3.7	2.6 2.9
Group 3, 11 cases	{				

recorded in group 2 representing from 1 to 2 milligrams of uric acid per 100 grams of blood are we believe well within the normal variations, but we are not prepared to say that they represent the full variations.

It is a curious and interesting fact that human blood contains several times as much uric acid as does the blood of any other mammal whose blood we have had the opportunity to examine. The exceptional position of man indicated by the high uric acid and extremely little allantoin in urine, as recently referred to by

* The blood was obtained in connection with the application of the "Wasserman reaction" and we wish to express our obligation to Dr. H. Adler for having given us a large number of samples.

Wiechowski⁷ and by Hunter and Givens,⁸ is thus again sharply indicated by the urea and content of the blood

For the urea and total non-protein nitrogen of human blood we do have figures representing strictly normal material. The blood was obtained from our medical students and from instructors in the department. It was drawn in the morning 3-6 hours after breakfasts which varied from "tea and toast" to "one egg, one pork chop, cereal, bread and cocoa."

TABLE 3

Total non-protein nitrogen and urea nitrogen in normal human blood
(Figures indicate milligrams per 100 grams of blood)

NAME	AGE	NON PROTEIN NITROGEN	UREA NITROGEN
O I	45	26	13
C F	26	25	12
H L	35	26	13
C E	35	25	13
J D	26	23	12
M D	28	25	11
H T	24	24	11
L R	22	26	11
A G	22	26	12
H B	22	22	11
L W	20	26	13
C S	25	22	11
E C	22	23	12
S E	22	25	12
J M	22	26	12
A S	23	24	13

From the figures for non-protein nitrogen and urea nitrogen in table 3 it will be seen that the former varies only between 22 and 26 milligrams and the latter only between 11 and 13 milligrams per 100 grams of blood. The narrowness of the range of variation of these figures is quite remarkable. It would seem that the perfectly normal kidney maintains a remarkably constant level of non-protein nitrogen and urea nitrogen in the blood. A "perfectly normal kidney" appears, however, to be the exception rather than the rule just as soon as one leaves the thoroughly

⁷ *Biochem Zeitschr*, xlv, p 433, 1910

⁸ *This Journal*, xiii, p 372, 1912

healthy adults and begins to work on any kind of "clinical material" The contrast between the normal non-protein nitrogen and urea figures of table 3 and the corresponding values obtained from syphilitics, as recorded in table 4, is rather appalling. Out of the 63 cases cited, and these were taken in the order of the examinations without any selection, there are but 13 whose values are not higher than the normal as represented in table 3. The

TABLE 4

Non-protein nitrogen and urea nitrogen in the blood of syphilitic patients
(Figures are given in milligrams per 100 grams)

NON PROTEIN NITROGEN	UREA NITROGEN	NON PROTEIN NITROGEN	UREA NITROGEN	NON PROTEIN NITROGEN	UREA NITROGEN
41	23	43	25	28	13
34	22	41	23	34	22
31	18	36	22	30	20
31	18	33	20	37	16
34	22	33	20	38	16
34	20	22	12	36	20
35	23	31	15	39	18
32	18	27	14	36	17
34	21	20	10	44	26
31	18	25	12	45	21
30	15	26	13	42	18
36	20	25	13	35	17
32	13	24	12	36	19
31	17	33	20	39	19
36	20	25	12	41	17
41	23	25	12	42	15
34	21	23	11	42	16
38	25	33	20	43	17
35	21	30	15	41	15
38	21	27	14	32	16
41	21	25	12	42	22

ages of these patients varied from fourteen to about seventy, the time of infection from a few weeks to about twenty years.⁹

That the impaired efficiency of the kidney indicated by the figures recorded in table 4 is not peculiar to syphilis is shown by corresponding values obtained from the insane. Out of 21 of these, the figures for which are given in table 5, there are only

⁹ We are indebted to Dr. Abner Post for this blood

7-10 whose nitrogen and urea figures are normal when judged by the standard values of table 3

In view of the striking difference in the condition of the blood of patients at hospitals and clinics as compared with that of strictly normal adults we were eager to see what kind of values would be obtained from patients suffering from recognized nephritis. The results obtained from eleven cases of "chronic nephritis" are given in table 6. Of these not a single one gave normal urea or non-protein nitrogen figures. The lowest figure obtained from nephritic blood is half again as large as the highest obtained from normal blood. On the other hand some of the nephritic patients

TABLE 5

Non-protein nitrogen, urea nitrogen and uric acid in unselected human blood

(The figures represent milligrams per 100 grams.)

NON-PROTEIN NITROGEN	UREA NITROGEN	URIC ACID	NON-PROTEIN NITROGEN	UREA NITROGEN	URIC ACID
43	18	1.2	32	17	2.4
27	14	1.4	24	13	2.0
24	18	1.3	35	18	2.6
50	22	1.2	62	46	2.1
40	21	3.0	36	19	2.8
52	32	1.6	28	16	2.0
24	13	2.0	32	18	2.5
50	26	1.4	36	19	1.7
36	19	0.8	28	16	2.4
28	16	2.0	32	19	1.8
34	18	1.5			

carry no greater accumulation of waste nitrogen in the blood than that found in some of the patients afflicted with syphilis or insanity. In how many of the latter it might have been possible for an expert clinician to make a diagnosis of chronic nephritis cannot now be determined.¹⁰

In the nitrogen and urea records of table 6 it will be noted that in the blood of one patient (No. 5, A. S.) the figures are nearly twice as large as the corresponding figures of any other in the table and nearly four times as large as the normal.

¹⁰ This paper represents only a preliminary survey of the field—a survey which we hope to follow up later by more exhaustive studies.

TABLE 6

Uric acid, urica nitrogen and non-protein nitrogen in nephritic blood
(Figures represent milligrams per 100 grams)

DESCRIPTION OF CASE	URIC ACID	NON PROTEIN NITROGEN	UREA NITROGEN
1 M P , "old lady", poorly nourished, blood pressure (sys), 200 Urine blood cells, casts	1 2	50	22
2 T F , age 65, "chronic nephritis," sys blood pressure, 220 Oedema, dyspnoea Urine albumin, casts and blood	2 7	40	19
2 Same patient one week later	2 5	38	19
3 J D , age 63, "chronic nephritis," arterio-sclerosis, aneurism aorta, very cyanotic	2 5	54	21
4 E H T , age 68, blood-pressure 260, no cardiac symptoms Urine trace of albumin, hyaline and granular casts	3 9	5 2	20
5 A S , age 45, blood-pressure 170, no oedema, skin very dark and variable in color Urine albumin, casts, blood	2 2	93	68
6 Ch W , age 57, rheumatic for years, no oedema, no dyspnoea Urine albumin, hyaline and granular casts	1 1	42	21
7 J F , age 55, oedema in the feet, dyspnoea Urine albumin, pus, no casts	2 1	41	21
8 Ch M , age 46, alcoholic, oedema in the feet, dyspnoea Urine hyaline and granular casts and blood cells	1 0	43	29
9 O D , age 38, "fairly acute nephritis", headache, vomiting Urine albumin, hyaline, granular and waxy casts	2 5	46	19
10 R , age 57, no cardiac symptoms, slight oedema, blood-pressure 180 Urine trace albumin, casts "Early stage of interstitial nephritis"	2 0	46	19
11 M L , age 51, blood-pressure 160 Urine casts, much albumin and pus Carcinoma of the breast and chronic nephritis—Dr Ordway	2 9	50	33

In connection with an extensive series of investigations of hum in blood in various diseases, but particularly nephritis, Strauss subdivided the cases studied into chronic, interstitial and chronic parenchymatous nephritis (with and without uremia). Strauss worked with blood serum, taking 100-200 cc of blood from each patient, and precipitated the proteins by means of acetic acid. It appears to us very improbable that the procedure employed by Strauss for removing the proteins could give uniform results. At least that is our experience in connection with the determination of uric acid in blood when we use a very similar method for removing the proteins. Traces of albuminous materials make no difference in our uric acid results, but would of course completely destroy the value of nitrogen determinations such as Strauss made. Strauss' results are nevertheless decidedly interesting. But his conclusion that the non-protein nitrogen in chronic interstitial nephritis averages 82 mgm per 100 cc of serum and 40 mgm in parenchymatous nephritis¹¹ is certainly not verified by our results reported in table 6.

The figures recorded in tables 5 and 6 are noteworthy in that they probably represent the first analyses on record where the uric acid, the urea and the total non-protein nitrogen have been determined in the same samples of human blood. *Further, from the figures we learn at once that there is apparently no relationship between the amount of uric acid and the amount of urea or total non-protein nitrogen in human blood.*

The above italicized generalization is, we think, of fundamental importance from a clinical as well as from a physiological point of view. The urea and total non-protein nitrogen in the blood must in the main be inversely proportional to the general efficiency of the kidney since the kidney represents practically the only outlet for the nitrogenous waste products. The significance of the uric acid is less clear. In the absence of some other plausible explanation, it, too, should occur in the blood in amounts inversely proportional to the ability of the kidney to remove it. Such a view was accordingly in earlier times used to explain gout (Garrod), a disease characterized by the accumulation of uric acid in the blood.

This simple explanation, naturally the first one to be thought of, has not been able to withstand the criticisms to which it has been submitted. To clinicians it has seemed incorrect because gout and general kidney inefficiency do not go hand in hand.

¹¹ *Die chronischen Nierenentzündungen* 1902 p 24

From a physiological standpoint it was far-fetched because it assumed a specificity of the kidney toward different waste products which was not warranted by anything that was known concerning the secretion of urine

A more modern explanation of the absence of any proportion between the uric acid and the other nitrogenous waste products in blood would be borrowed from the current teachings with regard to the destruction of uric acid within the body. Given some region, some tissue or organ, capable of destroying uric acid, and partial stagnation due to kidney inefficiency would result in the circulation of the uric acid laden blood over and over again through such a region and the accumulation of uric acid would be prevented. A few years ago no one would have hesitated to accept this last explanation. The work of Burian and Schur seemed to furnish a substantial experimental basis for it. Just now there is room for a certain amount of hesitancy because of the genetic relationship which has been discovered to exist between uric acid and allantoin in the urine (Wiechowski)

Allantoin is regarded as a final waste product whose precursor is uric acid. The urine of animals which contains little or no uric acid contains instead allantoin in amounts sufficient to account for the missing uric acid. In man, however, the allantoin excretion is almost nothing, amounting only to 10 or 15 milligrams or even less per twenty-four hours. The practical absence of allantoin in human urine has at once raised the issue whether uric acid is destroyed within the human organism. Wiechowski, Sivéén and Hunter and Givens¹² have taken the position that uric acid is probably a final waste product in man and is not destroyed by any human tissue. Frank and Schittenhelm adopt the older view of Burian and Schur that the destruction of uric acid within the human organism is quite extensive, and Brugsch and Schittenhelm¹³ have formulated a theory of gout based on a variable fermentative destruction of uric acid, the failure of which in gout is due to a defective cell metabolism and results in the accumulation of uric acid in the blood.

¹² For the recent literature on the subject see the article by Hunter and Givens already cited

¹³ *Loc cit*

If the amount of uric acid in human blood were always small, i.e., at the normal level, compared with the accumulation of the other nitrogenous waste products, there would hardly be room for any differences of opinion as to the ability of the human organism to decompose uric acid, notwithstanding the fact that it, unlike other animal organisms, does not convert the uric acid into allantoin. The situation is, however, rendered more complex by the well established fact that in certain kinds of human blood (gout and lead poisoning) there is an accumulation of uric acid.

Analyses of human blood characterized by a high uric acid content are recorded in table 7. For the time being we venture to call this blood "uric acid blood" in order to emphasize our finding that it is abnormally rich in uric acid without containing correspondingly large amounts of urea or other waste nitrogen. The distinctive character of this blood is we think unmistakable. The uric acid may reach the saturation point (in the form of urate of soda) and may be as abundant as in the blood of birds.¹

There is still a certain amount of dispute as to whether the uric acid elimination in gout is or is not appreciably diminished.

No one, however, claims that the uric acid output is increased above that found in the normal. This is important. The mere fact that the uric acid may accumulate in the blood of the gouty without being accompanied by an increased elimination constitutes definite proof that the gouty kidney is damaged with reference to its ability to eliminate uric acid.²

The hypothesis that there is normally a fermentative destruction of uric acid within the human tissues and that this destructive process is diminished in gout, as suggested by Brugsch and Schittenhelm, is hopelessly inadequate because the undisputed fact is that the gouty kidney fails to respond with an increased elimination of uric acid when there are noteworthy accumulations of uric acid in the blood. Further, the fact that some gouty kidneys are capable of eliminating the urea and other nitrogenous waste products almost or quite as well as strictly normal kidneys (see Nos 1 and 6, table 7) comes very near proving that the kidney activity is as selective as is the secretory activity of any other gland. In pure gout unaccompanied by any abnormal urea retention in the blood the kidney is damaged (so far as we yet know).

TABLE 7

Uric acid, urica nitrogen and non-protein nitrogen in "uric acid blood"
(Figures represent milligrams per 100 grams)

DESCRIPTION OF CASE	URIC ACID	NON PROTEIN NITROGEN	UREA NITROGEN
1 Dr H, age 53 Gout Several typical attacks during the past seven years No tophi Urine normal Last attack about six months ago	3.9	25	13
2 Mr B, age 65 "Gout and vascular nephritis"—Dr Pratt	5.5	52	36
3 Same case on a purine-free diet	3.4	40	18
4 Mr T, age 29 Gout and alcoholic gastritis—Dr Pratt	3.5	40	23
5 Mr S, "Pure gout, normal, very healthy, tophi"—Dr Pratt	4.4	30	15
6 Mr B, typical gout, last attack two years ago—Dr Joslin	5.2	28	13
7 Mr L, age 37, leukemia Spleen greatly enlarged Abdomen skin pigmented Oedema of feet—Dr Ordway	3.1	33	14
8 Same as 7 on a purine-free diet	2.8	44	20
9 Mr C, age 45, leukemia Large spleen Urine normal—Dr Ordway	4.1		
10 Mr X, age 45, lead poison, third attack Alcoholic for some years	4.7	50	31
11 Mr Y, Lead poison	4.8	52	32
12 Mr E, three attacks of pain in lumbar region during the past year, each time passed several small stones No arthritis no nephritis, no gout X-ray showed no stones in kidney—Dr Larrabee The stones contained over 95 per cent uric acid	5.2	30	13

only with regard to its function of removing down to the normal level the uric acid of the blood ¹⁴

The damage to the kidney resulting in the retention of uric acid and in the development of gout may be a very slight damage. The normal kidney clearly does not remove the uric acid from the blood as completely as the negative uric acid findings of previous investigators seemed to indicate. Normal human blood contains several times as much uric acid as they thought. Gouty blood on the other hand seems to contain less ¹⁵. Normal blood contains not less than from 1 to 2 or 2.5 milligrams of uric acid per 100 grams of blood. The blood in gout does not exceed 6 milligrams at least so far as our present experience goes. There is, however, no reason to suppose that a uric acid concentration of 4 to 6 milligrams per 100 grams of blood is very much more irritating or stimulating to the kidney than the somewhat more dilute solutions represented by normal blood. Disregarding the insolubility of uric acid the elevation of its threshold of elimination from 2 to 4 or 6 milligrams (per 100 grams of blood) is certainly a small one. Kidneys in which the threshold of elimination for urea has risen by 10 or 20 milligrams (per 100 grams) or even more are as we have seen (tables 4 and 5) extraordinarily common.

Such urea (and total nitrogen) retentions may represent latent or incipient nephritis, but in many, perhaps in most of these, the nephritis must remain latent or incipient, otherwise cases of recognized nephritis would be much more common than they are. Whether any recognizable effects on health may be traced to such urea retentions remains to be determined. In the case of uric acid it seems to be purely a matter of chance, purely a matter of insolubility, that corresponding or even smaller degrees of kidney insufficiency and slight uric acid accumulation should result in all the serious consequences involved in the development of gout.

¹⁴ In leukemia (see Nos 7, 8, 9, table 7) the cause of the high uric acid in the blood is an increased production, and the kidney responds by eliminating large quantities of uric acid.

¹⁵ From a consideration of the analytical technique employed by earlier investigators it is not difficult to see that they would entirely overlook moderate amounts of uric acid (1-2 mgm) yet would get (by weight or by nitrogen determinations) entirely too much if they found any at all.

We have said nothing in this communication concerning endogenous versus exogenous uric acid in blood—a distinction strongly emphasized by Brugsch and Schittenhelm in their papers on gout. The distinction is one long considered from a therapeutic dietetic standpoint. It may prove somewhat important from the standpoint of diagnosis, as well, in doubtful cases. As in the case of the urea the difference between the normal and the abnormal uric acid level in the blood should however be unmistakable without regard to reasonable variations in the purine intake.

The investigations of the accumulation of waste products in human blood in various diseases are being continued.

THE CHEMISTRY OF GLUCONEOGENESIS

II THE FORMATION OF GLUCOSE FROM VALERIANIC AND HEPTYLIC ACIDS

By A. I. RINGER

WITH THE ASSISTANCE OF L. JONAS

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(Received for publication, January 10, 1913)

In a previous communication¹ under this title, evidence was presented to the effect that in phlorhizinized animals, the administration of 10 grams of propionic acid, as ammonium or sodium salts, *per os* or subcutaneously, was followed by an increase in the glucose elimination which corresponded to all of the carbon of the propionic acid. The suggestion was then made that phlorhizinized animals have the power of quantitatively² synthesizing propionic acid into glucose. In this series of experiments the influence of the homologues of propionic acid are considered.

Methods

Female dogs were used in all the experiments. The urine was collected by catheter, and the bladder washed with distilled water at the end of every period. Merck's phlorhizin was used, and was injected in 2-gram doses three times per twenty-four hours, as recommended by Lusk.³ The following methods were used in the analyses: nitrogen, Kjeldahl-Gunning, ammonia, Folin, total acetone, Huppert-Messinger, aceto-acetic acid, Embden, glucose, the Allihn gravimetric method, also by polarization after clarifying the urine with basic lead acetate, β -hydroxybutyric acid, determined by Magnus-Levy's method. The β -hydroxybutyric acid results are relative, not absolute, for it was found that a considerable amount of

¹ A. I. Ringer. The Chemistry of Gluconeogenesis. I. The Quantitative Conversion of Propionic Acid into Glucose, this *Journal*, xii, p. 511, 1912.

² By quantitative, in this case, we understand the utilization of the entire molecule for the synthesis of glucose.

³ Graham Lusk. Phlorhizinglukosurie, *Ergeb. d. Physiol.*, xii, p. 315, 1912.

44 Formation of Glucose from Fatty Acids

phlorhizin was eliminated by the kidneys in the urine. This phlorhizin is extracted with the ether and has a specific rotation of -42.3° . The result of this investigation will be reported in the near future.

The effect of formic acid (HCOOH)

In the first table are tabulated the results of an experiment in which the animal received 11.5 grams of formic acid, subcutaneously, as sodium salt dissolved in 50 cc of water. There was no increase in the glucose, nor any increase in the D/N ratio. In experiment V, period V, the animal received a similar amount of formic acid, with similarly negative results. These two experiments show that *the diabetic organism does not possess the power of utilizing formic acid in the synthesis of glucose*.

The effect of normal butyric acid ($\text{CH}_3\text{-CH}_2\text{-CH}_2\text{-COOH}$)

In experiment V, period III, the animal received subcutaneously, 10 grams of normal butyric acid (Merck) dissolved in 50 cc of water and neutralized with sodium hydroxide. In experiment VI, period IV, the animal received 20 grams of the same butyric acid, administered in a similar manner. In neither experiment was there any increase in the glucose elimination. The aceto-acetic acid and β -hydroxybutyric acid, however, were increased quite markedly. During period V of experiment VI the animal was in deep coma, presenting all the typical symptoms of diabetic coma, and it ended in death.

The effect of normal valerianic acid ($\text{CH}_3\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-COOH}$)

In experiment VII, period II, 9.2 grams of normal valerianic acid (Kahlbaum), dissolved in 50 cc of water and neutralized with sodium hydroxide, were administered subcutaneously. The glucose elimination, which was 19.23 grams in the first period, rose to 24.13, in spite of a lower nitrogen metabolism in that period. The D/N ratio, which normally has a tendency to sink with the progress of the glucosuria, rose from 3.38 in the first period to 5.05 and 3.97 in the second and third periods, respectively. It is evident therefore that "extra glucose" was eliminated during periods II and III which did not find its origin in the catabolized protein. As no analyses were made during period IV of that experiment, we are forced to assume that the D/N ratio in

periods II and III would have remained at 3.38 had no valerianic acid been given. In calculating the "extra glucose" an error is thus introduced which makes the results a little too low. The extra glucose can be calculated by the following formula $EG = G - (N \times Q)$, EG stands for extra glucose, G stands for the value of the glucose eliminated, N stands for the value of the nitrogen, Q stands for the value of the assumed D/N quotient.

G in periods II and III is equal to $24.13 + 18.54 = 42.67$ grams

N in periods II and III is equal to $4.78 + 4.67 = 9.45$ grams

Q in periods II and III is assumed to be 3.38

$$EG = G - (N \times Q) = 42.67 - (9.45 \times 3.38) = 10.7$$

9.2 grams of normal valerianic acid give rise to 10.7 grams of extra glucose

In experiment IX, period II, the animal received 14.2 grams of normal valerianic acid administered as above. The glucose elimination rose from 18.28 grams in the fore period to 26.20 and 21.08 in the second and third periods respectively. The D/N ratio, which was 3.55 in the fore period, rose to 5.28 and 3.89 in the second and third periods.

Calculation of "extra glucose"

G in periods II and III is equal to $26.20 + 21.08 = 47.28$ grams

N in periods II and III is equal to $4.96 + 5.41 = 10.37$ grams

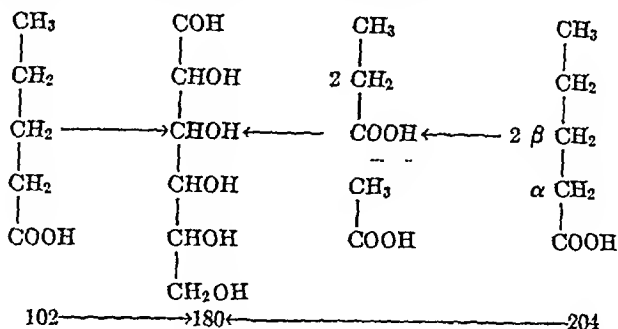
Q in periods II and III is assumed to be $\frac{3.55 + 3.64}{2} = 3.6$

$$EG = G - (N \times Q) = 47.28 - (10.37 \times 3.6) = 9.95$$

14.2 grams of normal valerianic acid give rise to 9.95 grams of extra glucose

These two experiments agree in showing that normal valerianic acid can be utilized in the synthesis of glucose. Although the extent of the utilization differs widely in these two experiments, we are justified, from the very nature of experiments on phlorhizinized animals, in accepting the maximal figures as the ones which show the maximum extent of synthesis. In experiment VII we obtained 10.7 grams of glucose from 9.2 grams of normal valerianic acid. Calculated per 10.0 grams of valerianic acid, the yield of glucose is equal to 11.6 grams. In experiment IX, 14.2 grams of valerianic acid yielded 9.95 grams of glucose, which gives for 10.0 grams of this acid 7.0 grams of glucose. How can

we assume this glucose to have arisen from the valerianic acid molecule? A glance at the relationship of the valerianic acid molecule to the glucose molecule may throw light on this



There are two possible ways in which valerianic acid may be converted into glucose. The first is by the utilization of all the carbons of valerianic acid in the upbuilding of the glucose molecule. In this case, 10.2 grams of the acid should give rise to 18.0 grams of glucose. This is not in accord with the findings in the experiments, and is besides highly improbable. The other possibility is that the products of decomposition of valerianic acid are synthesized into glucose. From the researches of Knoop, Embden, Dakin⁴ and others, it is well established that the normal fatty acids in the animal body undergo oxidation and cleavage in the β -position. Valerianic acid would then give rise to propionic acid, which has already been shown by the writer⁵ to be completely converted into glucose. Two molecules of valerianic acid would therefore be required for the formation of one molecule of glucose. Expressed in gram-molecular weight 10.2 grams of valerianic acid should give rise to 9 grams of glucose, which is in accordance with our findings. The conclusion is therefore justified that *valerianic acid gives rise to glucose in so far as it can give rise to propionic acid*.

The effect of normal caproic acid ($\text{CH}_3\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-COOH}$)

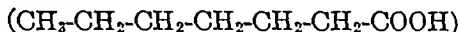
In experiment VIII, period II, the animal received 9.2 grams of normal caproic acid (Kahlbaum) as sodium salt. In experiment

⁴ H. D. Dakin, *Oxidations and Reductions in the Animal Body*, Longmans, Green and Company, 1912, contains the most recent review of the literature.

⁵ *Loc. cit.*

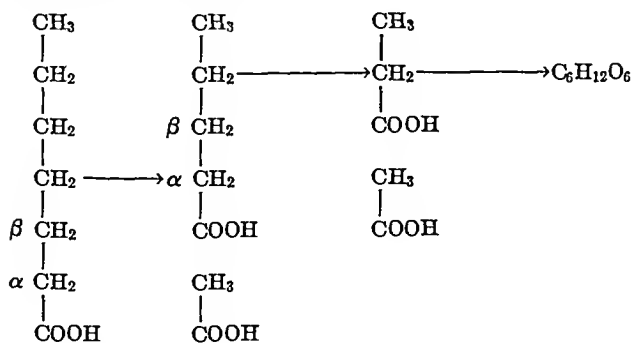
IX, period V, the animal received 10.3 grams of the same acid. In neither case was there any increase in the glucose elimination. The acetone bodies, aceto-acetic acid and β -hydroxybutyric acid were increased considerably, especially in experiment IX. These two experiments corroborate the findings of earlier investigators⁶ who found that caproic acid is a β -hydroxybutyric acid builder.

The effect of normal heptylic acid



In experiment IX, period VII, 13 grams of normal heptylic acid were administered subcutaneously. The glucose elimination in that period was increased, but to a lesser extent than after valerianic acid feeding. Assuming that all of the extra glucose came out in that period, then $E/G = G - (N \times Q) = 19.15 - (4.13 \times 3.75) = 3.67$ grams of extra glucose. In experiment X, period II, 13 grams of heptylic acid were administered as above. The glucose rose from 15.63 in the fore period to 20.99, the D/N ratio rose from 3.71 to 4.20.

$E/G = G - (N \times Q) = 20.99 - (5.0 \times 3.78) = 2.09$ grams of extra glucose. The amount of glucose derived from heptylic acid is indeed small, but an examination of the table convinces one that there is a decided increase, and that it can come from no other source. The heptylic acid no doubt undergoes oxidation and cleavage in the β -position, being converted into valerianic acid and finally into propionic acid, and this fraction of the heptylic acid molecule goes over into glucose.



⁶ H. D. Dakin *loc cit*

The yield of glucose in these two experiments was not as great as might theoretically be expected. Further experiments will be performed soon, with the hope of establishing a more exact quantitative relationship.

It is very evident from these experiments that, while the fatty acids with the even number of carbons, as butyric and caproic acids, give rise to aceto-acetic acid and β -hydroxybutyric acid, those with an uneven number of carbons give rise to glucose. The amount of glucose that arises diminishes with the size of the fatty acid molecule, and there is every reason to believe that it is only the three final carbons which contribute to the glucose formation. If this is proven to be true in the case of the higher fatty acids with C_{15} and C_{17} , it may be of the utmost value in the treatment of diabetes mellitus.

It is now very well established that aceto-acetic acid and β -hydroxybutyric acid find their origin mainly in the catabolism of the fatty acids with an even number of carbon atoms. 256 grams of palmitic acid in severe cases of diabetes may give rise to as much as 104 grams of β -hydroxybutyric acid. Should we find that the fats containing C_{15} or C_{17} (which are in progress of preparation now) are burned in the body, we may thus have a very efficacious means of combating acidosis. From the above experiments we may rightly conclude that the fatty acids with an uneven number of carbons undergo oxidation in the β -position, and thus they *cannot* possibly give rise to acids with an even number of carbons.

Very interesting, also, is the fact that, in every case in which gluconeogenesis takes place, it is associated with a coincident antiketogenic process. It is illustrated very markedly in the case of the propionic acid feeding.⁷

Experiments on the different phases of this problem and their bearing on diabetes are being carried on, and I beg to be permitted to reserve this field of research for a reasonable length of time.

SUMMARY

Experiments were performed on phlorhizinized dogs and the glucose, nitrogen, ammonia, acetone, aceto-acetic acid and β -hydroxybutyric acid eliminations were studied

I The administration of formic acid is not followed by any increase in the glucose elimination

II Butyric and caproic acids produce an increase in the aceto-acetic and β -hydroxybutyric acid eliminations, but no increase in the glucose

III Valerianic and heptylic acids give rise to glucose, in all probability because of the formation of propionic acid as an intermediary body in their catabolism, after undergoing β -oxidation

IV Attention is called to the fact that the fatty acids with an uneven number of carbon atoms give rise to glucose

The experimental data will be found in the following tables

Dec		EXPERIMENT IV <i>Twelve-hour periods</i>				
DATE 1912	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D N	REMARKS
22	I	10 0	5 41	20 26	3 74	11.5 grams formic acid as Na salt given subcutaneously in 50cc
22	II		5 08	18 50	3 64	
23	III		5 27	17 29	3 28	
23	IV		5 05	16 72	3 31	

EXPERIMENT V Twelve-hour periods

Dec	DATE 1912	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D N	NH ₄ N	ACETONE AND ACETO-ACETIC ACID	β HYDROXY-BUTYRIC ACID	REMARKS
	11	I	13 3	5 62	25 68	4 57	0 21	0 109	0 686	
	14	II		5 50	23 69	4 31	0 22	0 164	0 907	
	15	III	13 0	5 27	24 63	4 67	0 056	0 360	2 09	10 0 grams normal butyric acid (Merek) as Na salt given subcutaneously in one injection
	15	IV		5 34	21 35	4 00		0 162	1 16	
	16	V	12 8	5 86	22 15	3 78		0 155	1 81	11 5 grams formic acid as Na salt given subcutaneously
	16	VI		5 42	20 27	3 74		0 098	2 53	

EXPERIMENT VI Twelve-hour periods

Dec	I	II	III	IV	V	Dog in deep coma	Exitus
1	11 2	6 84	25 92	3 79	0 536	0 348	2 47
1		6 78	22 51	3 17	0 372	0 372	2 44
2		6 38	21 00	3 29	0 41	0 336	3 30
2	9 5	6 18	20 82	3 37	0 18	0 599	
3							20 0 grams normal butyric acid as Na salt given subcutaneously in two doses

EXPERIMENT VII Twelve-hour periods

EXPERIMENT VII Twelve-hour periods									
Nov	I	13 07	5 68	19 23	3 38	0 17	0 266	1 38	9.2 grams normal valerianic acid as Na salt given subcutaneously
2	II		4 78	24 13	5 05	0 158	0 227	1 50	
3	III	12 2	4 67	18 54	3 97	0 32	0 324	1 71	
3									

9 2 grams normal valeric acid as Na salt given subcutaneously

EXPERIMENT VIII Twelve-hour periods

Oct

DATE 1912	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D N	NH ₄ N	ACETONE AND ACETIC ACID	β HYDROXY BUTYRIC ACID	REMARKS
7	I	17 00	7 14	28 04	3 92	0 308	0 117	0 692	9 6 grams normal caproic acid as Na salt given subcutaneously
7	II		7 12	27 30	3 84	0 33	0 275	1 24	
8	III	16 46	6 65	25 93	3 89	0 35	0 575	2 13	

EXPERIMENT IX Twelve-hour periods

Nov

10	I	16 1	5 14	18 28	3 55	0 288	0 225	0 86	14 2 grams normal valeric acid as Na salt dissolved in 50cc of water given subcutaneously in one dose
10	II		4 96	26 20	5 28	0 139	0 100	1 41	
11	III		5 41	21 08	3 89	0 162	0 247	1 80	10 3 grams normal caproic acid as Na salt given subcutaneously
11	IV	14 70	5 35	19 48	3 64	0 198	0 293	2 74	
12	V		4 85	18 82	3 88	0 160	0 684	3 13	13 0 grams normal heptylic acid as Na salt dissolved in 75cc of water
12	VI	13 80	4 89	17 86	3 65	0 181	0 76	4 32	
13	VII		4 13	19 15	4 63	0 153	0 415	3 35	Lost
13	VIII	13 24	3 72	14 38	3 86	0 188	0 564	3 83	
14	IX		4 20	14 84	3 53	0 242	0 948		
14	X		3 90	14 79	3 79	0 220			

EXPERIMENT X Twelve-hour periods

Dec

DATE 1912	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D N	NH ₄ N	ACETONE β HYDROXY AND ACETO BUTYRIC ACETIC ACID	REMARKS
9	I	8.46	4.21	15.63	3.71	0.170	0.96	13.0 grams normal heptylic acid as Na salt given subcutaneously in two doses
9	II		5.00	20.99	4.20	0.106	1.91	
10	III		4.26	16.44	3.86	0.202	3.13	
10	IV		4.43	16.94	3.82	0.200	3.58	
11	V		4.18	15.23	3.64		2.84	

STUDIES IN COTTON SEED MEAL INTOXICATION I¹

PYROPHOSPHORIC ACID

By W A WITHERS AND B J RAY

WITH THE COLLABORATION OF R S CURTIS AND G A ROBERTS

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(Received for publication, January 2, 1913)

The injurious effect of the continuous feeding of cotton seed meal to calves and swine has been known for years. The cause has been ascribed by various investigators² to lint, oil, high protein content, a toxalbumin, choline, betaine, resin and decomposition products. Crawford³ in a preliminary paper published in March 1910 states that "The chief poisonous principle in certain cotton seed meals is a salt of pyrophosphoric acid." Crawford's conclusion is based upon the study of an extract obtained by digesting the meal at body temperature one day with pepsin and one day with pancreatin. He fed with a catheter extracts of cotton seed meal, these extracts corresponding to amounts of meal very much in excess of those which would be fed ordinarily. He made no study of the undissolved residues.

We began the study of the subject in 1908 and, since that time, have used many solvents in our efforts to extract the toxic substance. The undissolved residue having proved toxic in every case, we decided to investigate the residue undissolved by the pepsin-pancreatin treatment. We also tried other feeds which bear upon the question.

The animals selected were rabbits. Our normal daily feed for each animal was 15 grams of cotton seed meal or an amount of some fraction equivalent to 15 grams of meal. As the rabbits used averaged about 1.5 kilos the daily feed corresponds to 10

¹ This paper was read before the Biological Chemical Section of the American Chemical Society at the Washington meeting, December, 1911 (except the results of the last experiment on sodium pyrophosphate)

² *Exp. Sta. Record*, xxii, p. 502, 1910

³ *Journ. of Pharmacol.*, i, p. 547, 1910

grams per kilogram of initial live weight. The unconsumed feed was estimated and correction made. Each animal was allowed pea vines, cabbage leaves or other green feed each morning. We mixed molasses with the cotton seed meal and, when some fraction of the meal was being fed, the portion of meal removed was replaced by an equivalent weight of wheat bran. Six controls were run during the entire time of the experiment and all the control animals lived. The feeds, daily and total, are all calculated to the equivalent in grams of cotton seed meal per kilo of initial weight of animal.

Pepsin and pancreatin extract

Raw cotton seed meal was digested one day each with pepsin and pancreatin. The mass was filtered, and the filtrate concentrated to small volume. The solution was fed through a catheter in amounts corresponding to 200 grams of meal.

Nine rabbits were taken, varying in weight from 1461 to 2240 grams, average 1752. All lost in weight an average of 123 grams. Two were made very sick but survived, two showed no ill effect except loss in weight and five died. The amount fed corresponded to ten times our normal feed of cotton seed meal. The P_2O_5 in the feed was 2.22 grams.

Sodium pyrophosphate

Sodium pyrophosphate was prepared in the laboratory by igniting disodium phosphate. The product responded to the tests for pyrophosphate and for freedom from orthophosphates. 4.157 grams corresponded to 2.22 grams of P_2O_5 , the amount contained in the pepsin-pancreatin extract of 200 grams of meal. The pyrophosphate for each animal was dissolved in 65 cc of water and fed through a catheter.

One rabbit weighing 2070 grams died during the night following feeding. Another rabbit weighing 1456 grams, similarly fed, died thirty-one minutes afterwards.

The results of the preceding experiments indicate that the pepsin-pancreatin extract of 200 grams of meal is generally toxic when given at one feeding and that the $Na_4P_2O_7$ corresponding to the amount of P_2O_5 in that amount of meal is toxic if administered at a single feeding.

Cotton seed meal

Twelve rabbits were taken whose initial weights ranged from 970 to 2560 grams, the average being 1559 grams. All the animals died within from 8 to 21 days, average, 13 days. The average loss in weight was 379 grams. The total amount of meal consumed by each animal ranged from 105 to 225 grams, average, 157 grams. The total amount eaten was practically 100 grams per kilo of initial weight of the animal, making an average daily consumption of meal equivalent to 7.7 grams per kilo of animal. These figures indicate the degree of toxicity of the meal towards the animals under the conditions of the experiment. There were 2.76 grams of P_2O_5 in the average feed or 0.21 gram in the daily feed.

Sodium pyrophosphate corresponding to whole meal

Our cotton seed meal contained 1.76 per cent of P_2O_5 . If it were all in the form of pyrophosphate, 0.4157 gram of sodium pyrophosphate containing 0.222 gram of P_2O_5 would contain an amount equivalent to 12.5 grams of meal.

Four rabbits varying from 790 to 1550 grams, averaging 1117 grams, were fed daily 0.4157 gram of $Na_4P_2O_7$. Each animal gained in weight, the average gain being 243 grams. At the end of fifty-two days the feed was discontinued, all the animals being in good condition. For each kilo of animal the equivalent of the feed in cotton seed meal was a total of 582 grams, daily, 11.2 grams.

This feed furnished each animal daily with more pyrophosphoric acid than the amount received by each animal eating the raw meal, yet this feed was non-toxic and the meal toxic. This indicates that pyrophosphoric acid is not the cause of toxicity in cotton seed meal.

Our next step was to ascertain which was the more toxic portion of the meal, the aqueous extract, the pepsin-pancreatin extract of the residue undissolved by water or the residue undissolved after both of these treatments.

Aqueous extract of cotton seed meal

1260 grams of cotton seed meal were stirred at room temperature with 1050 cc of water and 10 cc of chloroform for twenty-

four hours. The solution was filtered, the liquid evaporated over a water bath to a syrup, poured over 240 grams of bran, mixed thoroughly, dried and fed to each animal in amounts corresponding to 30 grams of cotton seed meal daily—twice our normal feed. Six animals were taken. One animal died after 16 days, having lost 369 grams. The death being from causes other than the feed the observations are not included. The other five animals ranged in weight from 1137 to 1947 grams, average, 1400. The feed was discontinued after thirty-eight days, the animals having gained an average of 203 grams. There were 2.26 grams of P_2O_5 in the total average feed or 0.06 gram in the daily feed.

Pepsin and pancreatin extract

The residue left upon the filter in preparing the preceding feed was washed thoroughly with hot water and then digested at 40° for one day each with pepsin and pancreatin. The mass was filtered, the filtrate evaporated over a water bath to a syrup, poured over bran and dried. Each animal was fed daily the amount corresponding to 30 grams of cotton seed meal—double our normal.

Six rabbits were taken ranging in weight from 1516 to 1864 grams, average, 1683. At the end of thirty-eight days the feed was discontinued, the animals having gained an average of 73 grams each.

There were 5.22 grams of P_2O_5 in the total feed or 0.14 gram in the daily feed.

Residue undissolved by treatment with water, pepsin and pancreatin

The mass left after filtering off the solution from which the preceding pepsin and pancreatin extract feed was prepared was washed with hot water and dried. It was then finely ground in a mill. 8.8 grams, corresponding to 15 grams of meal, were mixed with bran and molasses and fed to each of six rabbits. The rabbits ranged in weight from 1374 to 1773 grams, average, 1592 grams. Five died in 14 to 16 days and the remaining one died at the end of 27 days—the average of all being 18 days. The average loss in weight was 325 grams. The total feed consumed corresponded to cotton seed meal equivalent to 116 grams per kilo initial average weight of animal, the average for a day being 6.4

grams per kilo The total P_2O_5 consumed by the average was 1.96 grams, the average for a day being 0.11 gram of P_2O_5 .

It is thus seen that the residue undissolved by the treatment of the meal with water, pepsin and pancreatin solutions is toxic, but both of the extracts, although they corresponded to almost three or four times as much meal, were non-toxic.

The total P_2O_5 in the toxic portion (1.96 grams) was less than in either of the non-toxic fractions.

Residue undissolved by pepsin and pancreatin

Cotton seed meal was digested as in our first experiment with pepsin and pancreatin. The mass was filtered and the residue was washed with water, dried and ground. 10.7 grams corresponded to 15 grams of meal. Three rabbits were taken, weighing 1860 and 2289 grams, average, 2124 grams. All lost in weight, the average being 552 grams, and all died in an average of 21 days—the range being from 15 to 27 days. The total feed per kilo of animal corresponded to an equivalent of 128 grams of cotton seed meal, the average daily feed being 6.1 grams per kilo. There were in the total average feed 1.40 grams of P_2O_5 , making a daily average of 0.07 gram.

This feed resembled the preceding feed closely, both in composition and results, and confirms our conclusions that the most toxic part of the meal is in the residue undissolved by pepsin and pancreatin.

We next prepared a feed which had an inappreciable amount of P_2O_5 and yet which was toxic. Our solvent was ammonium citrate solution.

Residue after citrate extraction

450 grams of cotton seed meal were extracted with 1500 cc. of a solution of ammonium citrate following the A. O. A. C. method for determining insoluble phosphoric acid. The mass was filtered, the residue washed and dried. 7.8 grams were the equivalent of 15 grams of meal.

Five rabbits were fed on this mixture. The weights ranged from 1350 to 2380 grams, average, 1602 grams. All lost in weight, the average loss being 438 grams. All died in from 17 to 29 days, the average being 21 days. The total feed equivalent in cotton

seed meal was 168 grams and the daily feed 80 grams per kilo of initial live weight

The total P_2O_5 consumed by each was 0.60 gram, a daily average of 0.03 gram. This feed was almost as toxic as the whole meal or the pepsin-pancreatin residue, although it contained but an insignificant amount of P_2O_5 .

Sodium pyrophosphate

The residue after ammonium citrate extraction contained a total of 0.60 gram of P_2O_5 in the amount consumed in twenty-one days. If all the P_2O_5 were in the form of pyrophosphate it would be the equivalent of 1.11 grams of $Na_4P_2O_7$. This amount dissolved in 60 cc. of distilled water was fed on January 17, 1912, at one feeding, through a catheter, to each of six rabbits, whose average weight was 1827 grams. The weights ranged from 1732 to 2037 grams. The average gain for each animal was three grams. The animals did not seem to experience any ill effects from the feed.

As the citrate residue, which proved toxic in 21 days, contained a total of 0.60 gram of P_2O_5 in the form of $Na_4P_2O_7$, and as this amount of P_2O_5 given at a single feeding to each of six rabbits proved harmless, the cause of the toxicity of the citrate residue is not pyrophosphoric acid.

SUMMARY

Experiments show that the pepsin-pancreatin extract of cotton seed meal is toxic generally to rabbits, when fed in amounts corresponding to from fifteen to twenty times the amount of meal normally fed, and that the pyrophosphoric acid corresponding to this extract is toxic if fed similarly.

The pepsin-pancreatin residue is more toxic than the aqueous or pepsin-pancreatin extracts of the meal—in fact it is the only one of the three fractions which is toxic under the conditions of the feeding.

A fraction of cotton seed meal containing a non-toxic amount of pyrophosphoric acid may be toxic.

The results indicate that pyrophosphoric acid is not the cause of toxicity of cotton seed meal.

THE INFLUENCE OF FUNCTION ON THE LIME REQUIREMENTS OF ANIMALS¹

By H STEENBOCK AND E B HART

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin)

(Received for publication, January 4, 1913)

A question which has baffled pathologists for a long time, and one which is not yet in a fair way of solution, is the cause of specific bone diseases known as rachitis, osteoporosis and osteomalacia, diseases which are all characterized by an impoverishment of the bones in inorganic substances. It is not desired to go into a discussion of the etiology of these pathological conditions, as a review of the literature will be found in various treatises,² but it is desired to correlate observations made in the study of these diseases with some experiments conducted with domestic animals designed to establish their minimum requirement of lime.

In regard to the amount of lime needed by an organism to enable it to maintain itself in lime equilibrium, we find no unanimity of opinion. It is evident that the lime requirements as well as the requirements for other inorganic substances vary with the physiological condition and activities of the animal. *A growing animal, a starving animal, a pregnant animal and a milk-producing animal, each has its specific drains upon the mineral nutrients.*

Undoubtedly an immature and growing animal demands a greater supply of lime in its ration than a mature one. Indeed the demand for lime in a dog has been found to stand in a direct relation to the rapidity of its growth.³ All investigators agree that with a growing animal a rachitic condition of the bones can

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² McCrudden *Archives of Internal Medicine*, v, p 596, 1910, Voit *Zeitschr f Biol*, xvi, p 55, 1880, Aron *Biochem Zeitschr*, xii, p 28, 1908, Dibbelt *Arbeit a d path Inst Tübingen*, vii, p 144, 1909

³ Aron and Sebauer *Biochem Zeitschr*, viii, p 1, 1908, Voit *Zeitschr f Biol*, xvi, p 55, 1880

be very readily obtained by feeding a low lime ration ⁴ With mature animals there is no such uniformity of opinion Dobbelt,⁵ working with a mature dog weighing 28 pounds, secured a positive balance of 0.1 gram of CaO over a ten-day period with a meat-lard diet supplying but 0.05 gram of CaO per day Heiss⁶ kept a mature dog weighing 8.8 pounds for a period of 308 days practically in lime equilibrium on a ration supplying 0.042 gram of CaO per day Forster,⁷ Voit,⁸ Müller⁹ and Kochmann,¹⁰ on the basis of their experimental data, maintain that such a low lime ration cannot satisfy the requirements of even a mature animal and present protocols showing negative balances on even high lime rations So far as we are aware, however, there are no tissue analyses at hand showing that the skeleton of a mature animal can be thus impoverished

With starving animals there is a continual loss of lime in the urine as well as in the feces Munk,¹¹ working with a dog, observed a loss of 0.176 gram in the feces and 0.029–0.096 gram in the urine daily for a ten-day period With the professional faster, Cetti, he¹² observed a loss of 4.08 grams of CaO in ten days and with Breithaupt, 0.561 gram in six days

With pregnant animals it is well known that a developing fetus constitutes an intense drain upon the lime supply of the mother, which may be sufficiently impoverished in this manner to show intense pathological symptoms ¹³

THE INTESTINAL FACTOR IN DETERMINING THE MINIMUM LIME REQUIREMENT OF ANIMALS FOR MAINTENANCE

Data collected from all sources show, without exception, that our domestic animals require a continual renewal of the supply of inorganic elements to repair the losses incurred in metabolism

⁴ Voit, Aron, McCrudden, Dobbelt *loc cit*

⁵ Dobbelt *loc cit*

⁶ Heiss *Zeitschr f Biol*, xii, p 165, 1876

⁷ Forster *Zeitschr f Biol*, ix, p 297, 1873

⁸ Voit *loc cit*

⁹ Müller *Zeitschr f Biol*, xx, p 327, 1884

¹⁰ Kochmann *Biochem Zeitschr*, xxxii, p 10, 1911

¹¹ Munk *Pflüger's Archiv*, lviii, p 309, 1894

¹² Munk *Virchow's Archiv*, cxxxi, Supplement, p 168, 1893

¹³ McCrudden *loc cit*

In all bone diseases where the animal may suffer extreme agony for want of lime it has been observed that during starvation and during periods of low lime feeding there is a continual lime loss, infinitesimal at times in the urine but always considerable in the feces. Even in prenatal life, lime accumulates in the meconium to a very considerable extent¹⁴. Why, in all these cases, the lime is not reabsorbed and re-utilized by the cells which demand it, is problematical as, theoretically, there is evident no chemical reason why the lime should not be available. It cannot be merely a question of solubility and that certain insoluble and therefore unavailable compounds are formed, in a salt solution, such as the body fluids, there is a continual redistribution in the combinations between acid and base radicals with the removal of any one component. It seems far more plausible to look upon this excretion of (unavailable?) salts as imposed on the animal by physical and physiological processes. Every cell, in the selection of its nutrients from a solution, has to perform a certain amount of work to overcome purely physical factors of solution and osmosis. Before this selection to the extent of the exhaustion of the desired element has taken place, the source of supply may have been removed and an excretion is observed under conditions of actual want. Specifically, the authors are inclined to concur with Dibbelt¹⁵ in his idea that the lime excreted in the feces even during rachitis has as its source normal secretions of the intestinal tract. Due to abnormalities in absorptive processes this lime is not reabsorbed to the usual extent and consequently makes its appearance in the feces. In support of this point of view we have the numerous observations that rachitis is usually preceded by visible digestive disturbances. Voit¹⁶ evidently was of a similar opinion as he said, "It is evident that the body is offered too small an amount of lime, not only in the case of an insufficiency of lime in the ration, but also when too small an amount of the ingested lime is absorbed through any cause as, for example, digestive disturbances or diarrhoea or defective assimilation from excessive feces-yielding nutrients". How digestive disturbances in themselves can call forth the excretion of lime into the intestine is not evident.

¹⁴ Müller *Zeitschr f Biol*, xx, p 327, 1887

¹⁵ Dibbelt *Habilitationschrift*, Tübingen, 1908

¹⁶ Voit *Zeitschr f Biol*, xvi, p 117, 1880

It seems far more probable that it merely inhibits absorptive processes. That, under certain conditions, excessive secretion of lime into the intestine cannot take place is not maintained as it is known that there is an actual secretion or excretion by this channel of numerous substances introduced into the animal body.¹⁷ The idea that the intestine may in certain respects function as a kidney is not a new one,¹⁸ but substances, foreign or native to the body, introduced in excessive amounts are not to be compared in their method of elimination with normally occurring processes. Indeed, Mendel and Benedict¹⁹ found that the injection of calcium salts was not always accompanied by increased calcium in the feces.

With domestic farm animals feeding on plant materials, it can readily be seen how the bulky digestive residues originating from these may hinder efficient absorption of secreted, lime-containing digestive juices. In this manner a feeding stuff high in indigestible material may require a higher initial lime content to enable it to maintain a positive lime balance for the animal than one composed of largely digestible materials. Again, the investigator is handicapped by lack of information on the form in which our inorganic materials are found in plant tissues. With oat straw,²⁰ all the lime has been found soluble in $\frac{N}{20}$ HCl, which gives us an idea of its solubility in the gastric secretion. Of its absorbability we cannot venture any statements. There are too many unknown factors which may influence the absorption of lime, of which the rôle of the various forms of *silica* in plant tissues may not be the least important.

Experimental

In view of the fact that no metabolism experiments have ever been conducted to determine the minimum lime requirements of farm animals as determined by intestinal excretion, it was considered desirable to secure such data with the pig and goat. The pig, above all animals, is most liable to be subject to an insufficiency of lime in the ration.

¹⁷ Mendel *Amer Journ of Physiol*, xi, p 5, 1904

¹⁸ Liebig *Chem Briefe*, 1845, pp 303-4

¹⁹ Mendel and Benedict *Amer Journ of Physiol*, xxv, p 23, 1909

²⁰ Unpublished data

EXPERIMENT I To secure these data a 75-pound pig was fed a ration initially very low in lime, but to which calcium phosphate was added in successively increasing amounts. Records of lime excretion were kept quantitatively for seven-day periods on a maximum consumption of the ration.

Composition of ration 1.8 pounds of rice, 0.2 pound of corn meal, 0.12 pound of wheat gluten.

The pig had previously been fed on the standard University ration, consisting of 30 parts corn, 30 parts oats, 30 parts middlings and 10 parts oil meal. On November 6 the ration was changed. Owing to the digestible character of the feed but little feces were excreted for a few days, which was mistaken for constipation. To remedy this 12 grams of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were added to the feed on November 12, and 15 grams, on November 13. On November 18 the first daily collection was made.

Though the intake of lime in the low-lime period was as low as was possible to secure, when supplying the necessary energy and nitrogen intake on unextracted plant materials, there was always observed a positive balance. In period V a negative balance appears to have prevailed, due merely to the carrying over in the gut of unexcreted lime from the preceding period. As has

TABLE I
Record of lime balance Daily averages in grams

	CAO IN FEED AND WATER	CAO IN FECES	CAO IN URINE	CAO RETAINED
I Low lime ration No additions Nov 18–Nov 24	0.245	0.208	0.012	+0.025
II Low lime ration + 2 grams $\text{Ca}_3(\text{PO}_4)_2$ Nov 25–Dec 1	0.897	0.207	0.016	+0.674
III Low lime ration + 4 grams $\text{Ca}_3(\text{PO}_4)_2$ Dec 2–Dec 8	1.673	0.347	0.013	+1.313
IV Low lime ration + 8 grams $\text{Ca}_3(\text{PO}_4)_2$ Distilled water Dec 9–Dec 15	2.916	0.688	0.023	+2.205
V Low lime ration Distilled water No additions Dec 16–Dec 20	0.203	0.708	0.013	−0.518
VI Low lime ration Distilled water No additions Dec 21–Dec 29	0.203	0.091	0.010	+0.102

been observed before,²¹ calcium phosphate offers a readily available source of lime to the pig. Here is brought out the additional fact of the tenacity with which lime once stored is retained, as no increased excretion was observed in the succeeding low-lime period. In fact, the lime content of the feces was even lower in the last low-lime period than in the initial. The reason for this is not evident unless the administration of the cathartic in the first period was an important factor. No excessive purging was observed.

EXPERIMENT II In experiment I was seen the failure of a low lime ration to cause a negative lime balance. In experiment II an attempt was made to reduce the lime intake still more by the use of properly acid-extracted plant materials of an initial low lime content. Commercial starches were found to average higher in lime than ordinary rice, which by extraction with dilute HCl and then repeated washing with distilled water to neutrality was lowered in lime content from 0.0103 per cent to 0.0049 per cent. Protein was supplied in the form of similarly extracted wheat gluten which was reduced to a lime content of 0.015 per cent. To prevent those physiological disturbances liable to occur on feeding a ration practically salt-free, a salt mixture containing 3 grams of K_2HPO_4 , 3 grams of NaCl and 1 gram of $MgSO_4 \cdot 7H_2O$ was added to the daily ration.

Pig I Growing pig. Initial weight, 111 pounds, final weight, 112 pounds. The composition of the daily ration was 943 grams of extracted rice, 0.0049 per cent CaO, 56 grams of extracted gluten, 0.015 per cent CaO, 7 grams of salt mixture.

TABLE II

Lime balance *Daily averages in grams* (Low lime ration fed, beginning February 9)

	CaO IN FEED	CaO IN FECES	CaO IN URINE	CaO RETAINED
February 17-23	0.054	0.097	0.030	-0.073

Pig II Mature pig. Initial weight, 312 pounds, final weight, 318 pounds. The composition of the daily ration was 2452 grams of unextracted rice, 0.0212 per cent CaO, 272 grams of extracted gluten, 0.0282 per cent CaO, 18.2 grams of salt mixture.

²¹ Hart, McCollum and Fuller. Research Bulletin 1, Wisconsin Experiment Station.

TABLL III

Lime balance Daily averages in grams (Low lime ration fed, beginning December 2)

	CAO IN FEED	CAO IN FECES	CAO IN URINE	CAO RETAINED
December 11-24	0 597	0 666	0 052	-0 121

Here in two specific cases the level of lime intake was brought below the amount necessary to obtain lime equilibrium. Of the specific forces which brought about these losses, both through the kidney and through the gut, nothing can be said at present. Whatever they are, they are as persistent in the pig as in other animals, which becomes evident when the intake is sufficiently reduced. From the data secured, a daily intake of 0.3 gram of CaO per 100 pounds body weight would cover these losses under the above conditions. What the requirements would be for more indigestible rations or for rations with a different accompanying salt content cannot be definitely stated.

THE FACTOR OF MILK PRODUCTION IN DETERMINING THE MINIMUM LIME REQUIREMENTS OF AN ANIMAL FOR ITS MAINTENANCE

In considering the influence of function upon lime metabolism not the least interesting and important is the factor of milk production. In a previous publication,²² as well as in subsequent verifications of these data,² it has been shown that with milk-producing animals, on certain rations, a very pronounced negative lime balance may be prevailing over extended periods of time. This, in its intensity, may assume much importance and necessitates further consideration of the principles underlying lime metabolism. In this problem consideration should be given to the relative efficiency of absorption of the mineral elements, the variations in the occurrence in the urine of various acids and bases and the interrelations prevailing among elements in their physiological capacities during and after lactation. All of these are of fundamental significance. It is hoped in some measure to throw further light on these relations in this article by

* Hart, McCollum and Humphrey, Wisconsin Experiment Station, Research Bulletin 5, 1909

²² Fingerling, *Landw. Versuchsstat.*, lxxv, p. 1, 1911

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a reconsideration of previous data as well as by the presentation of new observations

In regard to the interrelations prevailing among mineral elements in metabolism during lactation, data from this station, as well as those published by Rose,²⁴ give us some very interesting material. On a low phosphorus intake, with a heavy milk production, there invariably was observed the simultaneous occurrence of a large increase in the lime excreted in the urine over that of a previous period with a high phosphorus intake. A high urinary excretion of lime under conditions of actual want has never been previously observed except under conditions of prevailing acidosis where naturally an excessive demand is made upon the bases to maintain the neutrality of the tissues.

Acidosis as the cause of this high lime excretion is out of the question as the urine was always found distinctly alkaline in reaction. Here the high output of lime in the urine is to be considered as the direct result of an insufficient phosphorus assimilation at a time when the demand for this substance was augmented by milk secretion. As often observed in other connections, in an attempt to maintain the secretion of the mammary gland normal, the demands of the gland take precedence over the nutritive requirements of the body. Here, as a specific case in mineral metabolism, we have the mammary gland removing phosphorus from the body tissues in an endeavor to maintain its secretion normal in amount as well as in composition. This much our data show. As to the source of the phosphorus withdrawn we can merely speculate. *A priori*, we are led to believe that it was derived from skeletal tissues. In bone there is 1.72 times as much lime as in milk, in proportion to their phosphorus pentoxide content. Naturally, then, with the utilization of phosphorus from the skeleton for milk production, an excess of lime would be liberated and recovered in the excreta, which is exactly what was observed. Apparently all the excess of lime liberated made its appearance in the urine. If under any conditions the walls of the intestine function as an excretory organ for lime it is not evident in this connection. With the urine and also very probably the blood heavily laden with lime, we would certainly have reason

²⁴ Rose N. Y. (Geneva) Exp. Station, Technical Bulletin 20, 1912

to expect an increased excretion with the feces, but the amounts of lime in the feces do not show any consistent relation to the increased lime output in the urine. In summarizing the principles enunciated it may be briefly said that an inorganic substance such as lime may be excreted in increased amounts when in the excessive metabolism of another element it becomes a real waste product of metabolism.

To accumulate more data on the absorption and excretion of lime on different rations and with the animal serving different functions, a new series of experiments was carried out using the goat as the experimental animal. This made it possible to make all collections of excreta with the metabolism cage previously used in studying the lime nutrition of the pig.

Plan of the experiment. The goat was confined in the metabolism cage throughout the experimental period in connection with a study of creatinine metabolism during and after lactation. This necessitated the inclusion of some periods not immediately germane to the subject under consideration. The different rations enumerated were fed in succession and quantitative collections of the excreta made daily. Analyses of the excreta for lime were made in each period, when possible, only after a suitable lapse of time—usually five days—since feeding the preceding ration. Nitrogen determinations on the milk as well as on the excreta were made daily beginning April 17 and continued throughout the experiment.

A goat in full milk flow, producing about two quarts of milk daily on a corn, oat and clover ration, was selected as a suitable animal. Her quiet and tractable nature made her especially able to endure the long confinement in the metabolism cage, with little inconvenience and that shown only toward the close of the period. But in spite of the prevailing opinion that a goat has no epicurean tendencies it was found impossible to obtain a consumption of the ration of bran, rice, wheat gluten and oat straw on which the cow, previously referred to, had been fed. Though for various reasons it was highly desirable to duplicate the ration as nearly as possible, a considerable reduction in the amount of bran and the addition of some corn was found necessary to secure consumption.

The periods involve two phases of experimentation. The first two are records of the animal in full milk. In period III the milk

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flow was gradually being reduced. The periods following III are records with complete or almost complete cessation of mammary function

Rations fed

PERIOD I *April 4-May 10*

7 5 pounds of wheat bran	}	0 030 per cent CaO
45 0 pounds of corn meal		
30 0 pounds of rice meal		
17 5 pounds of gluten meal		
25 0 pounds of oat straw		0 472 per cent CaO

An approximate average of 1 6 pounds of grain and 0 4 pound of straw consumed daily

PERIOD II *May 11-May 17*

Same ration as in I with straw intake doubled

An approximate average of 1 6 pounds of grain and 0 8 pound of straw consumed daily

PERIOD III *May 18-May 31*

8 0 pounds of corn meal	}	0 028 per cent CaO
5 0 pounds of rice meal		
1 0 pound of wheat bran		
1 0 pound of gluten meal		
10 0 pounds of oat straw		0 472 per cent CaO

An approximate average of 1 2 pounds of grain and 0 8 pound of straw consumed daily

PERIOD IV *June 1-June 11*

6 0 pounds of corn meal,
6 0 pounds of rice meal,
8 0 pounds of oat straw

An approximate average of 1 2 pounds of grain and 0 8 pound of oat straw consumed daily

PERIOD V *June 12-July 2*

Straw only

An approximate average of 1 2 pounds of straw consumed daily

PERIOD VI *July 3-July 29*

Same ration as in Period I

An approximate average of 0 8 pound of grain and 0 2 pound of straw consumed daily

PERIOD VII *July 30-August 17*

Allowed to graze during the day and fed in addition 1 2 pounds of oats daily

PERIOD VIII *August 18-September 5*

Same ration as in Period I and VI

An approximate average of 0 8 pound of grain and 0 2 pound of straw consumed daily

TABLE IV

Record of lime balance in the several periods and daily averages

	CAO IN FEED	CAO IN WATER	CAO TOTAL INTAKE	CAO IN EX- CRETA	CAO ABSORBED	CAO IN MILK	CAO IN EX- CRETA* AND MILK	CAO BALANCE
	grams	grams	grams	grams	grams	grams	grams	grams
I April 11- May 10	1 07	0 18	1 25	1 10	+0 15	2 97	4 07	-2 82
II May 11-17	1 92	0 15	2 07	1 48	+0 59	2 74	4 22	-2 15
III May 18- 23	1 86	0 12	1 98	1 45	+0 53	1 71	3 16	-1 10
V June 17-28	2 56	0 10	2 66	2 52	+0 14	0 13	2 65	+0 01
VI July 7-29	0 55	0 06	0 61	1 54	-0 93	0 03	1 57	-0 96
VIII August 24-Sept 5	0 60	0 06	0 66	0 42	+0 24	0 00	0 42	+0 24

* Lime in excreta as tabulated does not include the urinary excretion. This varied from traces to a maximum of 0.04 gram in very exceptional cases. Data on this were accumulated only for a total of about seven days in each period and were judged insignificant in the above connection. No tendencies to vary the urinary excretion in either direction by any of the rations were apparent.

TABLE V

Nitrogen balances

	N IN FEED	N OUTGO TOTAL	N BALANCE
	grams	grams	grams
I April 20-April 26	126 07	127 36	- 1 29
April 27-May 3	135 71	131 68	+ 4 03
May 4-May 10	135 71	130 66	+ 4 05
II May 11-May 17	142 50	133 55	+ 8 95
III May 18-May 24	86 15	93 96	- 7 81
V June 15-June 21	19 38	36 64	-17 27
June 22-June 23	20 35	28 55	- 8 29
VI July 6-July 12	77 55	68 65	+ 8 90
July 13-July 19	69 13	61 69	+ 7 44
July 20-July 26	67 85	65 65	+ 2 20

Discussion Period I We have here a verification of results obtained with the cow namely, milk production on a low lime ration results in a pronounced negative lime balance. Here also we have again the appearance of a large amount of fecal lime, the origin of which is not evident. A slight excess of lime intake over that excreted is observed, which for the sake of convenience is designated "lime absorbed." The table on nitrogen balance shows an actual retention in spite of the large milk secretion. The

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absorption of nitrogen and lime as indicated by the fecal excretion do not parallel. Apparently they are separate and distinct functions.

Period II Essentially this is a continuation in results of the data in period I. The increased amount of straw in the ration, in spite of the assumed tendency for it to depress the reabsorption of lime containing secretions by the increase in fecal bulk, has actually increased the absorption of lime. Other factors must have come into play. These data are somewhat invalidated by the briefness of the period of observation, with the omission of the usual interval between periods. The fecal lime, however, was relatively constant in amount throughout this period, which would not have obtained if such factors had influenced the character of the data. It seems safe to conclude that the lime from oat straw was available to the goat.

Period III This period marks the beginning of a reduction in the amount of protein fed, in an attempt to reduce the milk production for the later periods of observation. The utilization of lime and its balance does not show any new characteristics.

Period IV Here we have a still further reduction in the protein ingestion, but as no analyses for lime were made this period need not occupy our attention.

Period V To get a clearer insight into the availability of lime from straw this period ought to offer very satisfactory data, as the ration consisted exclusively of oat straw. We observe, however, but a very small utilization of the lime. The animal is only saved from a continuation of the impoverishment of its tissues in lime by the reduction in the milk flow. Upon comparison of the lime retention with the N retention one would say that here is to be found the explanation for the small positive lime balance observed. With a pronounced deficit in nitrogen, bone tissue could not be built up, consequently there was not any immediate demand for lime, in spite of the previous depletion.

Period VI In continuation of this line of reasoning one would, in view of the generous N retention, expect a corresponding lime retention. The animal had been depleted in lime as well as nitrogen and now with the first opportunity a restoration of these elements would be expected. For nitrogen, this occurred, though the animal, already weighing scarcely 68 pounds, 11 ounces, con-

tinued to lose in weight until at the close of this period she weighed but 63 pounds. For lime there was a steady and consistent negative balance day after day with no indications of a change. The urinary lime excretion was not increased. Physical symptoms of osteomalacia were not observed although the animal showed a slight stiffness in the hind quarters, which was not surprising after such close confinement in a metabolism cage for a period of three months.

The feces were hard and dry and small in bulk, a slight failing in the appetite as well as a slight unresponsiveness to call or change in its environment began to be apparent. No definite results could be expected by longer continued observation under these conditions.

Period VII On a ration of June grass and oats recovery speedily followed. In the course of two weeks with a gain in weight of 4 pounds all symptoms indicative of approaching collapse were dispelled. On the supposition that the change in physiological condition might influence her lime metabolism additional data were collected.

Period VIII In view of the fact that no reliable results on the retention of nitrogen can be obtained over a short isolated period, no record of the nitrogen balance was kept. All indications were that the animal was in a good nutritive condition. Her eyes were bright, her senses acute and she showed a uniform gain in weight of 2 pounds during the period. Regular consumption of the ration was secured, and the feces were again of a normal consistency, as the table shows, a retention of lime now took place as a direct result of the accompanying change in the condition of the animal. This transformation is truly remarkable and from the standpoint of mineral metabolism stands unparalleled in the experimental field. From a nutritive condition which would ultimately have led to the occurrence of physical symptoms of osteomalacia, there was brought about a complete restoration to the normal by a temporary change in the ration. That the change of intake of lime was responsible for this transformation is out of the question, as the former ration, for maintenance, left little to be desired from the inorganic standpoint. Some subtle change in the trend of metabolism was instituted by the temporary variation in feed intake, as well as environment, which now enabl

the animal to assimilate lime, formerly unavailable. From this point of view osteomalacia is not to be considered primarily a physiological disturbance brought about by the unavailability of lime in the source of supply, but rather the result of inefficient absorption of lime from the gut due to a complication of numerous factors. What these are we cannot venture to say. There was not a cessation of all absorptive processes in the intestine since in period VI we noted a generous utilization of the nitrogen from the ration in spite of the excessive loss of lime. An independence of specific assimilative functions could not possibly be more positive than in this connection. It is either a peculiar coincidence or else an important observation that with a pig in an experiment previously reported²⁵ a slight negative lime balance was likewise observed simultaneous with the excretion of hard and dry feces.

CONCLUSIONS

1 The level of lime intake necessary for maintenance is dependent upon the functional activity of various organs of the body. A daily intake of about 0.3 gram of CaO per 100 pounds body weight covered the metabolism losses of a mature barren pig. From 0.4 to 0.5 gram of CaO per 100 pounds body weight covered the metabolism loss of a mature dry goat. The figures are not absolute and general, but will vary with the character of the ration.

2 The mammary gland during its activity constitutes a severe drain upon the skeletal lime supply during periods of insufficient lime assimilation. During periods of insufficient phosphorus assimilation, it indirectly causes a waste of lime from the skeleton.

3 An allowance of 1 gram of lime in the ration per pound of milk produced by a goat or cow should theoretically be ample. This, of course, is in addition to the maintenance requirement. But at least twice the above amount would be safer, due to the large losses of lime in the intestine accompanying increased food consumption.

4 The walls of the intestine with their normal secretions may cause the loss of a sufficient amount of lime to appreciably lower its "coefficient of digestibility" during periods of sufficient lime

²⁵ Research Bulletin 30, Wisconsin Experiment Station

ingestion When such conditions are complicated by physiological disturbances a large negative balance of lime over an extended period of time may result

5 Under normal conditions with a low lime ingestion the usual intestinal losses may in themselves be the cause of a negative lime balance

6 Intestinal and urinary losses of lime do not parallel With very heavy intestinal losses the urinary excretion may remain unchanged

7 A liberal assimilation of nitrogen does not necessarily imply an assimilation of lime even when the animal's supply of lime is considerably depleted These are separate and distinct functions of the alimentary tract

8 A perverted lime metabolism which ultimately would end in an extreme impoverishment of the skeleton in lime may be merely the result of other physiological disturbances

THE EFFECT OF A HIGH MAGNESIUM INTAKE ON CALCIUM RETENTION BY SWINE ¹

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The experiment to be described in this paper relates to the influence which the addition of magnesium salts and of magnesium salts plus phosphates to the diet may exert upon calcium metabolism. They may also be interpreted as throwing some light upon the general problem of the mutual relationships which exist among the mineral constituents of the diet, considered from the standpoint of animal nutrition.

Many experiments are on record which indicate the existence of important biological relationships between calcium and magnesium. For example, Loew² showed that magnesium could serve in the nutrition of plants only in the presence of calcium salts. Loeb³ found the contractile reaction in jellyfish induced by magnesium to be inhibited by calcium. Meltzer and Auer⁴ proved that the effects of calcium on muscle and nerve in rabbits could be reversed by the injection of magnesium salts. In dogs, Mendel and Benedict⁵ observed that the injection of calcium salts was followed by increased elimination of magnesium in the urine; similarly injection of a magnesium salt increased the urinary output of calcium. Malcolm⁶ published evidence tending to show

¹ Published by permission of the Director of the Agricultural Experiment Station

² Loew U S Dept of Agric, Div Veg Path and Physiol, Bulletin 18

³ Loeb this *Journal*, 1, p 427, 1905-06

⁴ Meltzer and Auer *Amer Journ of Physiol*, xvi, p 400, 1908

⁵ Mendel and Benedict *Amer Journ of Physiol*, xxv, p 1, 1909

⁶ Malcolm *Journ of Physiol*, xxxii, p 183, 1905

that ingestion of magnesium salts may cause a loss of calcium from adult animals and may hinder its deposition in the young Forbes,⁷ basing his considerations upon these data, is inclined to believe that the cause of "bran disease," "shorts disease" and "millers horse rickets" is to be found in an excessive proportion of magnesium to calcium in bran and shorts

Similarly, the influence of phosphates in the diet upon calcium metabolism is indicated in the work of Ingle⁸ who attributed the cause of a bone disease, manifested chiefly by impoverishment of the skeleton in ash, to a disproportionate content of lime and phosphorus pentoxide in the feeding stuff Dibbelt⁹ observed the loss of 12.7 grams of lime in the feces alone from the body of a small dog when 5 grams of Na_2HPO_4 were added daily to its meat and fat diet Bertram,¹⁰ working with a goat, likewise observed loss of lime from the body when Na_2HPO_4 was added to its ration

In order to obtain data bearing upon Forbes' hypothesis mentioned above as well as to gain further information concerning the influence of variation in the mineral content of the ration upon calcium excretion, the following experiment was carried out

The subject of the experiment was a pig, weighing at the beginning of the experiment 166 pounds, at the end, 156 pounds Quantitative collection of excreta was made possible by confinement in a metabolism cage previously described by McCollum and Steenbock¹¹ The bran ration fed throughout the experiment consisted of corn, 1.3 pounds, wheat bran, 2.4 pounds, oatmeal, 0.3 pound, and contained 0.134 per cent CaO , 0.820 per cent MgO , 2.924 per cent P_2O_5 Of this ration 1 pound 11 ounces was consumed daily

Although the proportion of MgO to CaO in the ration was high, it seemed advisable to accentuate the probable effect of the

⁷ Forbes Ohio Experiment Station Bulletin 213, 1909

⁸ Ingle *Journ of Agric Science*, III, 1908, *Journ of Comp Path and Ther*, XXI, XXII, 1907-08

⁹ Dibbelt *Arbeits d Geb d path anat Inst Tubingen*, VII, p 559, 1911

¹⁰ Bertram *Zeitschr f Biol*, XIV, p 340, 1878

¹¹ McCollum and Steenbock Research Bulletin 21, Wisconsin Experiment Station

magnesium in one part of the experiment by adding magnesium as the chloride, in another, magnesium was added as the sulphate. Later, in view of the depressing influence which phosphoric acid exerts upon the solubility of calcium and magnesium salts, a period was included in which the bran ration was supplemented by the addition of both magnesium salts and a soluble phosphate. Potassium phosphate was used in amount sufficient to combine with all the magnesium salts added to form tertiary magnesium phosphates.

RECORD OF CALCIUM EXCRETION

DATE	CaO IN RATION	CaO IN FECES	CaO IN URINE	CaO IN EXCRETA	CaO RETAINED
	grams	grams	grams	grams	grams
January <i>Bran ration only</i>					
14	1 67	1 64	0 010	1 650	
15	1 67	1 47	0 059	1 529	
16	1 67	1 49	0 000	1 490	
17	1 67	1 37	0 000	1 370	
18	1 67	1 40	0 030	1 430	
Daily average.	8 35	7 37	0 099	7 469	+0 881
	1 67	1 47	0 019	1 490	+0 170
January <i>Bran ration + 6 08 grams of MgO as MgCl.</i>					
19	1 67	1 38	0 155	1 535	
20	1 67	1 41	0 276	1 686	
21	1 67	1 42	0 221	1 641	
22	1 67	1 56	0 269	1 829	
23	1 67	1 39	0 278	1 668	
24	1 67	1 24	0 262	1 502	
25	1 67	1 49	0 229	1 719	
26	1 67	1 69	0 258	1 948	
27	1 67	1 44	0 305	1 745	
28	1 67	1 51	0 342	1 852	
29	1 67	1 20	0 292	1 492	
30	1 67	1 70	0 253	1 953	
31	1 67	1 13	0 067	1 197	
Daily average	21 71	18 56	3 207	21 767	-0 057
	1 67	1 42	0 246	1 673	-0 003

78 Calcium Retention after Magnesium Ingestion

RECORD OF CALCIUM EXCRETION—Continued

DATE	CaO IN RATION	CaO IN FECES	CaO IN URINE	CaO IN EXCRETA	CaO RETAINED
	grams	grams	grams	grams	grams
February <i>Bran ration only</i>					
1	1 67	1 21	0 188	1 398	
2	1 67	1 75	0 091	1 841	
3	1 67	1 09	0 063	1 153	
4	1 67	1 33	0 045	1 375	
5	1 67	1 40	0 061	1 461	
6	1 67	1 52	0 085	1 605	
7	1 67	1 52	0 065	1 585	
8	1 67	1 52	0 088	1 608	
9	1 67	1 27	0 089	1 359	
Daily average	15 03	12 61	0 775	13 385	+1 645
	1 67	1 40	0 086	1 487	+0 18
February <i>Bran ration + 6 08 grams of MgO as MgSO₄</i>					
10	1 67	1 35	0 291	1 641	
11	1 67	1 42	0 093	1 513	
12	1 67	1 35	0 406	1 756	
13	1 67	2 03	0 720	2 750	
14	1 67	1 58	0 371	1 951	
15	1 67	1 34	0 299	1 639	
16	1 67	1 22	0 341	1 561	
Daily average	11 69	10 73	2 521	12 811	-1 121
	1 67	1 47	0 360	1 830	-0 160
<i>Bran ration + 6 08 grams of MgO as MgSO₄ + K₂HPO₄ to form Mg₃(PO₄)₂</i>					
17	1 67	1 66	0 340	2 000	
18	1 67	1 34	0 200	1 540	
19	1 67	1 67	0 114	1 784	
20	1 67	1 44	0 108	1 548	
21	1 67	1 83	0 115	1 945	
22	1 67	1 50	0 115	1 615	
23	1 67	1 65	0 180	1 830	
24	1 67	1 35	0 096	1 446	
25	1 67	1 26	0 121	1 381	
26	1 67	1 53	0 123	1 653	
27	1 67	1 95	0 128	2 078	
Daily average of last nine days	18 37	17 18	1 640	18 820	-0 450
	1 67	1 57	0 122	1 697	-0 027

The data presented in the table show conclusively that a large amount of bran in the ration exerted no inhibiting action upon the animal's ability to assimilate lime from the ration. It is true that the amount retained was not very large, but, in any case, with the large losses normally occurring with the fecal excretion such a positive retention is to be viewed as a reliable index. With bran the bulk of the fecal excretion is large, which in itself may exert an intensified depreciating effect on the actual amount of lime absorbed from the ration itself.

The addition of magnesium salts did not increase the fecal lime excretion. Accompanied by the simultaneous addition of potassium phosphate a slightly increased lime content of the feces was observed. This cannot be overlooked as there is a possibility that the ingestion of the potassium phosphate may merely have changed the path of excretion for the lime. That this is not entirely the case is seen by the decrease in the total lime elimination during this period.

In the urine, with each period where magnesium salts alone were added, a remarkable increase in the urinary lime excretion is observed. This can be attributed to no other factor than magnesium salts, as the data from day to day are exceedingly consistent. The addition of soluble phosphates reduces this increment but not to its former level. Of the exact relations existing here the data offer us no information. But, in itself, it is truly remarkable that potassium phosphate should be able to counteract, even in part, the injurious action of the magnesium salts. Further, during this period there was not an increased magnesium excretion through the intestine.

Here, in the interrelations prevailing among mineral elements, we may have an explanation of some of the numerous anomalies that have been observed in connection with mineral metabolism experiments, otherwise well controlled. Though the effect of organic nutrients upon mineral metabolism is not to be underestimated, it undoubtedly is true that, in the condition of the digestive system and in the interrelations prevailing among mineral elements, we have two factors of primary importance operative in mineral metabolism.

CONCLUSIONS

1 The unfitness of bran as a bone producing feeding stuff is to be attributed to its low content of lime capable of absorption, rather than to a disproportionate content of calcium to magnesium

2 Magnesium salts added to a pig's ration increased the calcium elimination in the urine The fecal calcium excretion was not influenced

3 Soluble phosphates, as di-potassium acid phosphate, decreased the increased calcium excretion brought on by the addition of magnesium salts

4 The fact that the relation of phosphorus to calcium and magnesium in our grains is high, with the probable formation in the tract of magnesium phosphate and its excretion by way of the intestine, would help to explain the difference in the action of magnesium ehloride or sulphate and the magnesium normal to grains

5 The interrelations existing between mineral elements are important factors for consideration in studying the specific rôle of a mineral element in animal nutrition

ON THE EXTREMES OF VARIATION OF THE CONCENTRATION OF IONIZED HYDROGEN IN HUMAN URINE

By LAWRENCE J HENDERSON AND WALTER W PALMER¹

(From the Chemical Laboratory, Massachusetts General Hospital)

(Received for publication, January 20, 1913)

In a recent paper² we have shown that the variations of hydrogen ion concentration in urine, when studied in a large number of instances, are not without physiological and pathological significance, and it is evident that the latitude of variation of this quantity is a fundamental factor in the regulation of the neutrality of the animal body. For this and other reasons, it is of interest to determine the extreme variations of acidity and alkalinity which can arise in man.

Considering the large amount of acid which is daily removed from the body (equivalent to 600-700 cc of $\frac{N}{10}$ acid) and the enormous quantities which may be excreted in diabetic acidosis (equivalent to 6 liters of 0.1 N acid or more) it is obvious that higher acidity than that which is to be observed among the cases of a large general hospital is not to be expected under any circumstances, for it is not possible safely to introduce into the body more than a small fraction of such quantities of acid. Experiment bears out this view.

To study the effect of acid ingestion on the hydrogen ion concentration, 10 grams of monosodium phosphate were given at a single dose and the urine collected in separate specimens at frequent intervals thereafter.

The variation in hydrogen ion concentration from hour to hour in the various cases is only slightly greater than that which occurs in normal cases without monosodium phosphate intake. In every case, however, there is found a slight increase of hydrogen ion concentration. The greatest difference is observed in case 7,

¹ Henry P. Walcott, Fellow in Clinical Medicine, Harvard Medical School.

² L. J. Henderson and W. W. Palmer, this *Journal*, vol. p. 393, 1913.

TABLE I

Concentrations of ionized hydrogen after the administration of acid phosphate *

NUMBER	TIME ACID SOLUTION PHOSPHATE GIVEN	TIME OF COLLECTION AND HYDROGEN ION CONCENTRATION OF URINE AFTER NaH_2PO_4 INGESTION										
		7 00 a m	9 00 a m	10 00 a m	11 00 a m	12 00 noon	1 00 p m	2 00 p m	3 00 p m	6 00 p m	10 00 p m	morning following
1	a m											
1	9 00		6 85	6 50	6 00	6 15		6 50	7 00		6 50	6 30
2	9 00		5 85	5 60	5 70	5 85	5 40	5 30	6 50			
3	9 00		5 40	5 70	5 85	5 30	5 15	5 30	5 60		5 70	
4	9 00		5 15	5 30	5 00		5 15	5 15		5 60	5 70	7 00
5	7 30	5 60			5 30				5 70	6 50	5 70	5 30
6	7 00	5 60							5 30	5 70	6 30	5 00
7	9 00		6 70	7 00	6 30	6 15	5 30	5 40				6 30
8	7 00	5 50					5 30			5 50	5 70	5 40

Expressed as negative logarithms see Henderson and Palmer *loc cit*

where the initial value was low, 6 70, and at 1 00 p m, four hours after the ingestion of monosodium phosphate, it was 5 30, representing a twenty-five fold increase in acidity. Administration of larger quantities of acid phosphate, or of hydrochloric acid, produces similar effects and never, in our experience, causes acidity of the urine as great as that which is common in many pathological conditions.

On the other hand the body is not known ever to produce bases in considerable quantity (except ammonia to neutralize an excess of acid) hence it is natural to expect that the administration of alkali in the normal individual may produce a urine more alkaline than that which is otherwise to be found. This view also has been confirmed by experiment.

TABLE II

NUMBER	+ H WITHOUT ALKALI	FIRST DAY WITH ALKALI		SECOND DAY WITH ALKALI	
		Sodium bicarbonate	+ H	Sodium bicarbonate	+ H
		grams		grams	
1	6 15	24	8 00	40	8 70
2	6 00	20	8 70		
3	6 30	12	7 40		
4	6 70	20	8 30		
5	6 00	24	8 30	40	8 70
6	6 85	8	8 50	16	8 50
7	6 50	12	8 30	24	8 50
8	6 70	12	8 70		

The effect of the ingestion of large amounts of sodium bicarbonate was studied in young adults. As far as possible the sodium bicarbonate was taken between meals and the hydrogen ion concentration was determined on twenty-four-hour specimens of urine.

The results of a single dose of sodium bicarbonate on the hydrogen ion concentration are given in the following table. As far as possible a specimen of urine was obtained at the time of administration of the sodium bicarbonate and hourly specimens obtained for several hours thereafter.

TABLE III

NUMBER	SODIUM BICARBONATE	TIME SODIUM BICARBONATE GIVEN	TIME OF COLLECTION OF SPECIMEN OF URINE AND HYDROGEN ION CONCENTRATION					
			10 00 a m	11 00 a m	12 00 noon	1 00 p m	2 00 p m	3 00 p m
	grams	a m						
1	4	10 00	7 40	8 30	7 48	7 48	7 40	5 85
2	8	10 00	5 40	8 50	8 30	6 50	6 50	7 40
3	12	10 00	5 30	8 70	8 70	8 70	8 70	8 70
4	8	10 00	7 40	8 50	8 70	8 50	8 50	8 50
5	8	10 00	5 85			8 70	8 70	8 30
6	8	10 00	6 70	7 48	8 70	8 50	8 70	8 50

In no instance were we able, by the administration of sodium bicarbonate, to obtain a more alkaline urine than one with a hydrogen ion concentration of 8.70, which is slightly more alkaline than a solution of disodium phosphate of concentration about 0.01 N. From the foregoing data it is evident that the reaction of the urine may be pushed down to a certain degree of alkalinity with comparative ease, beyond this point, even after the administration of large amounts of alkali, the reaction of the urine does not change.

The highest acidity which has thus far been observed, exceeds 4.70, the highest alkalinity, 8.70. This corresponds to a range of 1/10,000 in the concentration of hydrogen and hydroxyl ions, and amounts on the one hand to the acidity of a solution consisting of a trace of free phosphoric acid together with monosodium phosphate, on the other hand, to the alkalinity of a solution of disodium phosphate of concentration about 0.01 N.

The variations of the relative amounts of acid and alkali which accompany such variations in reaction are very large and, together with ammonia excretion, measure the protective power of the kidney

Ten cubic centimeter samples from twenty-four-hour amounts of urine from normal individuals and 10 cc of a standard solution having a hydrogen ion concentration of 4.70 (highest observed acidity) were introduced into separate 250 cc flasks, diluted with distilled water to approximately 250 cc and five drops of a 2 per cent aqueous solution of sodium alizarine sulphonate added. Sufficient sulphuric acid of concentration 0.1 N was added to the flask containing urine to bring its reaction to that of the standard solution. A second titration was carried out with a fresh 10 cc sample of urine and 10 cc of a standard solution with a hydrogen ion concentration of 8.70 (lowest observed alkalinity), similarly diluted, ten drops of a 1 per cent alcoholic solution of phenolphthalein added, and the reaction of the urine carried to that of the standard solution by addition of potassium hydrate of concentration 0.1 N. In each instance the number of cc of $\frac{N}{10}$ acid or alkali to bring the reaction of the total amount of urine to the reaction of the standard solutions was calculated

TABLE IV

NUMBER	AMOUNT	+ H	TOTAL ACID	TOTAL ALKALI	ACID + ALKALI	RATIO OF ACTUAL TO POS- SIBLE ALKALI IN URINE
	cc		cc	cc	cc	
1	2200	6.15	185	246	431	0.43
2	1200	6.30	380	568	948	0.40
3	1300	6.30	284	545	829	0.34
4	1300	5.85	285	705	990	0.29
5	1500	7.00	411	212	623	0.61
6	2500	6.70	500	680	1180	0.43
7	2175	6.50	425	675	1100	0.39
8	1000	5.85	188	385	573	0.33
9	1920	6.85	432	430	862	0.50
10	1300	6.70	271	316	587	0.45

Average (alkali + acid)

812

Maximum (alkali + acid)

1180

Minimum (alkali + acid)

431

It thus appears that the possible variation in the amount of alkali in union with the acids of normal urine is ordinarily about 0.5-1.0 gram-molecule. In pathological conditions, when the acids are increased, this quantity also is necessarily larger.

The actual variation in normal individuals is evidently at least nearly 0.5 gram-molecule.

In the most acid urines which we have observed, the urinary acids, in addition to phosphoric acid and acid sodium phosphate, are free to a considerable degree, as follows: hippuric acid, 8 per cent, acetoacetic acid, 11 per cent, lactic acid, 12 per cent, β -oxybutyric acid, 45 per cent, uric acid, 91 per cent.

In the most alkaline urines all these acids are almost completely combined with base, and any free carbonic acid must be accompanied by a very large quantity of sodium bicarbonate—at least one hundred times its concentration. Such is the explanation of the large variation in relative amounts of acid and alkali.

We wish to thank Dr. Frederick C. Shattuck for making this work possible. Thanks are also due to the members of the visiting staff and others of the Massachusetts General Hospital for their kindness in supplying clinical material.

THE ESTIMATION OF CREATININE AND CREATINE IN DIABETIC URINES

By ISIDOR GREENWALD

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(Received for publication January 21 1913)

That acetone and acetoacetic acid react with picric acid and sodium hydroxide with the production of a colored solution, similar to that produced by creatinine under the same conditions, has long been known. The extent of their possible interference with the estimation of creatinine, by the Folin method, has not been clearly determined. Folin¹ states that the reaction is given by hydrogen sulphide, acetone, acetoacetic acid and its ester, which, if present, may easily be removed. No directions are given. Klercker,² who used very large amounts of acetone in his experiments, found that acetone caused a rapid fading of the color. According to van Hoogenhuyze and Verploegh³ the readings obtained in the presence of acetone are at first too low, but as the color due to the acetone fades rapidly, normal readings are soon obtained. Similar results are reported by Krause,⁴ who also investigated the action of acetoacetic acid. He found that the readings were too low. Wolf and Osterberg,⁵ on the contrary, reported that the addition of 1 per cent of acetoacetic ester was without influence upon the determination of creatinine. Rose⁶ used acetoacetic acid in concentrations up to 0.25 per cent and obtained correct readings if the solutions were allowed to stand three or four minutes after dilution before making the readings.

¹ Folin *Zeitschr f physiol Chem* vii p 223, 1904

² Klercker *Biochem Zeitschr*, iii, p 45, 1907

³ van Hoogenhuyze and Verploegh *Zeitschr f physiol Chem*, lvi, p 161, 1908

⁴ Krause *Quart Journ Exp Physiol* iii p 289, 1910

⁵ Wolf and Osterberg *Amer Journ of Physiol* xlviii, p 71, 1911

⁶ Rose *this Journal* vii, p 73 1912

In connection with an investigation into the creatine and creatinine metabolism in diabetes, it became necessary to ascertain if acetone and acetoacetic acid in the amounts present in diabetic urines would interfere with the estimation of creatinine. This was found to be the case. Although the addition of a small quantity of acetone (0.5 per cent) to normal urine does not greatly affect the readings obtained, such addition to diluted urine or to urine containing but little creatinine, interferes very decidedly. The presence of acetoacetic acid also makes all the readings unreliable. The figures given in Table I are typical of the results obtained.

TABLE I

The effect of acetone and acetoacetic acid upon the estimation of creatinine (10 cc. of the urine were diluted to 250 cc. after the addition of the picric acid and sodium hydroxide solutions)

NUMBER	NATURE OF URINE	ADDITION	READINGS
1	Normal, diluted		11.06
		0.5 per cent acetone	10.30 fading to 11.6
		Acetoacetic acid*	12.00 fading to 12.4
2	Normal, diluted		10.10
		Acetoacetic acid*	10.80 fading to 13.0
3	Muscular dystrophy		10.50
		Acetoacetic acid*	13.00

* Enough of a freshly prepared solution of acetoacetic acid was added to produce a Gerhardt reaction comparable to that given by a urine then under examination in this laboratory.

In order to make an accurate estimation of creatinine, by the Folin method, it is therefore necessary either to remove the interfering substances or to precipitate the creatinine, determine the creatine in the filtrate and subtract the amount found from the sum of the creatine and creatinine. Boiling the urine, as recommended by Rona,⁷ was first attempted. It was found that the urine from a severe case of diabetes required boiling for at least five minutes to free it of acetoacetic acid. A number of experiments were made with urines from patients with muscular dystrophy. Creatinine was determined in the usual manner. A volume of urine equal to that taken for the determination of creatinine was boiled for five minutes, cooled and the creatinine estimated. In every instance the readings were much lower than

⁷ Rona *Handbuch d. biochem. Arbeitsmethoden*, III, p. 788, 1910.

TABLE II

The effect of boiling the urine upon the estimation of creatinine in the presence of creatine

NUMBER	VOLUME OF URINE	DILUTION	READINGS	
			Unboiled	Boiled
	cc	cc	mm	mm
1	25	500	6 87	5 20
2	25	1000	8 13	6 97
3	25	250	7 23	6 12
4	25	300	8 55	7 35

in the control estimation (Table II) The method was therefore rejected

Fairly good results were obtained by precipitating the creatinine with sulphuric and phosphotungstic acids, filtering, freeing an aliquot portion of the filtrate of the acids with barium hydroxide, filtering, precipitating the excess of barium in an aliquot portion of the filtrate with sodium sulphate, adding hydrochloric acid, evaporating and completing the determination of creatine in the usual manner. A urine from a case of muscular dystrophy contained 0.276 gram preformed, and 0.881 gram total creatinine per liter. To some of this urine, 4 per cent of dextrose was added and the determination of creatine carried out as described above. Calculated as creatinine, 0.611 gram was found. In another urine the content of preformed creatinine was 0.822 gram and of total creatine, 1.200 grams per liter. The amount of creatine, as estimated by this method after the addition of 4 per cent of dextrose to the urine, was 0.382 gram, calculated as creatinine, per liter. As a rule, however, the agreement was not so close as in these instances. The method is not altogether satisfactory.

At the suggestion of Dr. S. R. Benedict, an attempt was made to determine the creatinine after extraction of the acetoacetic acid with ether and subsequent aeration to remove the ether and acetone. This was found to be very successful. The apparatus used consisted of an ordinary Soxhlet extraction apparatus, a short, wide test-tube and a funnel-tube. In principle it was the same as that described by Saiki.⁸ After two hours the urines were found to be free of appreciable amounts of acetoacetic acid. For

⁸ Saiki, *this Journal*, vii, p. 21, 1909.

the determination of creatinine the following procedure was adopted 25 cc of the urine are measured into the extraction tube, 10 cc of concentrated hydrochloric acid added and the mixture extracted with ether for two hours The contents of the tube are then washed into an aerometer cylinder and aerated for one hour To reduce foaming, a few drops of toluene may be added and the aeration continued until this has been removed The estimation is then carried out in the usual manner except that, because of the dilution, 30 cc of the picric acid solution and 10 cc of the sodium hydroxide solution are used Sodium hydroxide equivalent to the hydrochloric acid used must also be added The results of a number of estimations in urines from patients with muscular dystrophy are summarized in Table III In order to eliminate the influence of suggestion, most of the determinations were made by two observers, one comparing the colors, the other reading the scale

That dilute hydrochloric acid does not, under these conditions, effect an appreciable conversion of creatine into creatinine is also

TABLE III

Comparison of results obtained by direct estimation of creatinine in urines containing creatine and after addition of acetoacetic acid and its extraction with ether

NUMBER	DIRECT	AFTER ADDITION OF ACETOACETIC ACID AND EXTRACTION	TIME ELAPSED BETWEEN THE ADDI- TION OF THE HYDRO- CHLORIC ACID AND THE ESTIMATION
	mgm per liter	mgm per liter	hours
1	235	235	
2	226	229	
3	472	472	
4	793	797	4
5	613	610	
6	232	228	3
7	494	486	6
8	494	498	18
9	434	432	3 5
10	434	439	8
11	434	434	4
12	395	398	4
13	424	430	20
14	541	537	5
15	359	359	7

indicated by the following experiments. Forty-eight milligrams of creatine were dissolved in 100 cc of 0.5 N HCl solution. At intervals the liquid was tested for creatinine by Jaffé's reaction and also with phosphotungstic acid. Even after four days there was no precipitation on the addition of phosphotungstic acid nor was the color produced with picric acid and sodium hydroxide different from that of the alkaline picrate solution. On another occasion, 0.119 gram of creatine was dissolved in 100 cc of water and 4 cc of concentrated hydrochloric acid. After six hours at a temperature between 37° and 40°, the solution gave no precipitate with phosphotungstic acid and only a slight reaction with picric acid and sodium hydroxide solutions.

TABLE IV

Comparison of readings obtained in the direct estimation of creatinine in diabetic urine and after extraction with ether
(25 cc of urine diluted to 250 cc before reading)

NUMBER	DIRECT	AFTER EXTRACTION
	mm	mm
1	9.3	7.80
2	9.4	8.30
3	14.7	9.20
4	11.1	7.12
5	11.6	6.86

A few examples of the results obtained by this method, as compared with the original Folin method, are given in Table IV. The urines were obtained from a patient with a severe form of diabetes. The figures in the first column were obtained after waiting until the readings no longer changed appreciably within three or four minutes. This required from five to ten minutes. The color may continue to fade slowly for a much longer time as is shown by two experiments, the results of which are given in Table V. The readings did not remain constant until about thirty minutes had elapsed.

Rose⁹ has called attention to the low readings obtained, in the presence of dextrose, in the Benedict-Myers modification of the Folin method for the determination of creatine. He has proposed

⁹ Rose *loc cit*

the substitution of phosphoric acid for the hydrochloric acid. Apparently he did not use the original Folin method. As is shown in Table VI, this is quite satisfactory. If care be taken to prevent undue concentration, the effect of dextrose, even in a concentration of 5 per cent, is barely appreciable, although the urines contain so little creatinine that the dilution before reading is only 250 or 300 cc.

TABLE V

Readings obtained in the estimation of creatinine in diabetic urine by the Folin method

	I	II		I	II
Immediately	13 0	6 9	After twenty minutes	18 2	10 2
After five minutes	14 7		After thirty minutes	19 0	10 2
After ten minutes	16 8	9 0	After forty-five minutes	19 0	
After fifteen minutes	18 0	9 3			

TABLE VI

The effect of dextrose upon the estimation of creatine by the Folin method (10 cc. of diluted normal urine made up to 250 or 300 cc. before reading)

NUMBER	DEXTROSE ADDED	READINGS	NUMBER	DEXTROSE ADDED	READINGS
	gram	mm		gram	mm
1		9 00	5		7 93
	0 3	8 95		0 5	7 80
2		6 88	6		8 07
	0 4	6 83		0 5	7 98
3		5 57	7		9 90
	0 5	5 45		0 5	9 74
4		7 75	8*		7 01
	0 5	7 80		0 5	7 08

* Dog urine

In this laboratory, the conversion of creatine into creatinine is generally accomplished in 50 cc. flasks with short necks. The condensation in the neck prevents the loss of any appreciable amount of water but the acetone is almost completely removed. Three urines, which contained, respectively, 14.6, 10.5 and 17.6 mgm. of acetone in 10 cc., after being heated with hydrochloric acid as in the determination of creatine, contained only 1.7, 1.0

and 1.9 mgm of acetone in 10 cc. Such small amounts of acetone are without appreciable effect on the readings obtained.

The Folin method is so simple that it seems to be preferable to the much more troublesome procedure recommended by Rose. The only advantage of the latter is that conversion is completed a little sooner, but the time actually required for the manipulation is greater. Except when a large number of determinations are to be made at the same time, it offers no advantages over the Folin method.

SUMMARY

In urines containing acetoacetic acid or acetone, creatinine may be accurately estimated by the Folin method only after removal of these substances. A method of accomplishing this is described. Dextrose, in concentrations up to 5 per cent, is without appreciable effect upon the estimation of creatine by the Folin method.

ON THE COLORIMETRIC DETERMINATION OF URIC ACID IN URINE

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(Received for publication, January 27, 1913)

By means of the new colorimetric method for the determination of uric acid in urine recently described by Folin and Macallum¹ it is possible to make a reliable uric acid determination in about fifteen minutes. The method consists essentially in evaporating the acidified urine to dryness and extracting from the residue certain polyphenol compounds which give a blue reaction with the uric acid reagent.² The insoluble uric acid is left behind and is then determined colorimetrically. Unfortunately this method is not directly applicable to all kinds of urine. Urines of certain animals as for example the rat and cat and also certain kinds of pathological human urine (urines containing albumin or sugar) leave on evaporation such coatings of inert materials that it is practically impossible to remove quantitatively the interfering polyphenol compounds by means of alcohol-ether mixtures or indeed by any other solvent which does not also dissolve uric acid.

In their preliminary paper Folin and Macallum³ described a different method for separating the uric acid from the disturbing polyphenols. The uric acid was precipitated by means of ammo-

¹ This *Journal*, *xiii*, p. 363, 1912

² The urine was acidified with oxalic acid before the evaporation. This produced some urea oxalate which protected the uric acid mechanically without interfering appreciably with the alcohol-ether extraction. Recently C. Farmer and F. B. Grinnell have made a series of extractions after acidifying with 1 cc. of 1 per cent monosodium phosphate solution. Judging from the results obtained with pure uric acid solutions as well as with normal urines the results so obtained are rather more satisfactory with pure uric acid solutions and equally satisfactory with urine.

³ This *Journal*, *xi*, p. 265, 1912

niacal silver solutions more or less as in the Ludwig-Salkowski method. The great drawback to this procedure was the fact that in the presence of the silver the blue color obtained on adding the uric acid reagent was so very transient in character that correct results could be obtained only by working with a very high degree of speed and uniformity of procedure.

Having solved the problem of how to make use of ammoniacal silver solution as a reagent for isolating uric acid from blood in a condition suitable for colorimetric determinations⁴ we had, of course, in that procedure one that could hardly fail to be applicable to all kinds of urine. The general method thus evolved necessitates the use of a small centrifuge but it is otherwise as rapid, convenient and accurate as the method described by Fohn and Macallum for normal urine. At all events because of its general applicability to pathological urines the method would seem to merit a separate description, especially as we are now able also to describe a method for the preparation of a satisfactory standard.

Standard uric acid solutions for the colorimetric determination of uric acid

No other detail in the colorimetric determination of uric acid has presented such difficulties as has the problem of finding a suitable standard. Theoretically the most satisfactory standard for any color comparison is the color obtained with a known amount of the substance which is to be determined. In all our work we have used fresh uric acid solutions for this purpose because no other stable substance has been found to give exactly the same color as that given by uric acid. It is distinctly tedious, however, to make up standard uric acid solutions every day or every few days. The standard which was obtained by adding an excess of uric acid to an exact amount of the uric acid reagent⁵ was not entirely satisfactory, the chief drawback being the distinctly greater rapidity with which the color of such solutions fade as compared with the solutions obtained by adding an excess of the reagent.

⁴ This Journal, xii, p. 469, 1913.

⁵ This Journal, xii, p. 367, 1912.

By combining uric acid with formaldehyde we have at last obtained solutions of uric acid which act like ordinary pure uric acid solutions with reference to the quality and stability of the color produced with an excess of the uric acid reagent. The solutions so obtained seem to keep their strength indefinitely. We have now had several different solutions for three months, have kept them in the light and in the dark, in cold places and in warm ones, and in no case have we observed any diminution in the color value obtained with a given excess of the uric acid reagent. The uric acid formaldehyde compounds do not possess more than a small fraction of the reactivity corresponding to the uric acid contained in them and their value in terms of uric acid must be determined just like that of any unknown uric acid solution but the important thing is that the active fraction, whatever it is due to, remains constant.⁶

The uric acid formaldehyde solution is prepared as follows. One gram of uric acid in a volumetric liter flask is dissolved by means of an excess of lithium carbonate (200 cc of a 0.4 per cent solution). To the solution are added 40 cc of 40 per cent formaldehyde solution and the mixture is shaken and allowed to stand for a few minutes. The clear solution is acidified by the addition of 20 cc of normal acetic acid and the whole is diluted up to the liter mark with water. The solution should remain perfectly clear and the next day (but not before) it can be standardized against a freshly prepared lithium carbonate solution of uric acid. The color produced by 5 cc of the solution corresponds very nearly to the color obtained from 1 mgm of uric acid. The colorimeter reading obtained for the solution when thus compared against 1 mgm of pure uric acid is, of course, thereafter to be used as the standard value corresponding to 1 mgm of uric acid.

The new uric acid method

From 1 to 2 cc of urine are measured into an ordinary centrifuge tube by means of a modified Ostwald pipette. A sufficient amount of distilled water is then added to bring the volume of

⁶ Uric acid formaldehyde compounds were first prepared by Tollens (*Ber d deutsch chem Gesellsch*, xxv, p 2514, 1897). The solubility of these compounds has long been known but their stability in aqueous solutions has evidently not been recognized before.

the liquid in the tube to about 5 cc, six drops of 3 per cent silver lactate solution, two drops of magnesia mixture, and a sufficient amount (10-20 drops) of concentrated ammonium hydrate to dissolve the silver chloride are then added. The tube is now centrifuged for one or two minutes, the supernatant liquid poured off and to the residue in the bottom of the tube are added five or six drops of freshly prepared saturated hydrogen sulphide water and one drop of concentrated hydrochloric acid, and the tube is placed in a beaker of boiling water until all excess of hydrogen sulphide has been driven off.

As hydrogen sulphide gives a blue color with the "uric acid reagent" care must be taken to obtain its complete removal. To determine whether this has been accomplished one drop of 0.5 per cent lead acetate solution should be added to the contents of the tube after the latter has remained in the water bath for about five minutes and if any hydrogen sulphide still remains a dark brown precipitate will be formed. If this condition be obtained the tube should be returned to the water bath for a further period of heating.

When the tube has been cooled, add 2 cc of the uric acid reagent, 10 cc of saturated sodium carbonate solution, transfer to a 50 cc volumetric flask and make up to volume. The color comparison is then made in the usual manner against the color obtained from 5 cc of the standardized uric acid-formaldehyde solution (or a freshly prepared pure uric acid solution).

In the case of urines containing much albumin it will be found that after the addition of hydrogen sulphide the solution obtained is invariably of a brownish tint, which interferes with the color comparison, and thus makes accurate readings very difficult. This difficulty may be overcome by adding to the hot solution (after the removal of all hydrogen sulphide) from two to ten drops of a 10 per cent sodium acetate solution.

This procedure has also been found useful in the determination of uric acid in blood where the same trouble is met with when as occasionally happens the protein has not been entirely removed.

Unless albumin be present sodium acetate should not be added either in blood or urine analysis as its presence tends to give slightly low results.

The uric acid determinations recorded below show that the new method is capable of giving excellent results in the presence of large amounts of albumin or sugar. The figures represent grams of uric acid per liter of urine.

NO OF URINE	FOLIN-SHAFFER METHOD	NEW METHOD	NO OF URINE	FOLIN-SHAFFER METHOD	NEW METHOD
1	0 49	0 50	12	0 70	0 76
1 + 10% serum		0 52	12 + 20% serum		0 75
2	0 49	0 49	13	0 60	0 57
2 + 10% serum		0 50	13 + 5% dextrose		0 57
2 + 20% serum		0 47	13 + 10% dextrose		0 57
3	0 84	0 80	14	0 82	0 83
3 + 10% serum		0 82	14 + 5% dextrose		0 83
3 + 20% serum		0 80	14 + 10% dextrose		0 86
4	0 60	0 60	15	0 58	0 62
4 + 10% serum		0 59	15 + 5% dextrose		0 62
5	0 69	0 71	15 + 10% dextrose		0 62
5 + 10% serum		0 70	16	0 65	0 70
6	0 47	0 49	16 + 5% dextrose		0 70
6 + 40% serum		0 50	16 + 10% dextrose		0 69
7	0 16	0 17	17	0 68	0 71
7 + 40% serum		0 17	17 + 5% dextrose		0 70
8	0 47	0 44	17 + 10% dextrose		0 71
8 + 20% serum		0 45	18	0 47	0 46
9	0 65	0 67	18 + 5% dextrose		0 46
9 + 2% egg albumin		0 66	18 + 10% dextrose		0 47
10	0 44	0 42	19	0 87	0 82
10 + 20% serum		0 43	19 + 5% dextrose		0 80
11	0 48	0 50	19 + 10% dextrose		0 81
11 + 20% serum		0 49			

ON THE NATURE OF THE IODINE-CONTAINING COMPLEX IN THYREOGLOBULIN

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In this paper are given the results of an attempt to determine the nature of the active complex in the iodine-containing active principle of the thyroid gland. Although the nature of this group was not determined, the quantitative physiological results here reported serve to establish certain predicted and other unexpected facts and to eliminate certain hitherto considered probabilities.

The problem was taken up both by analytical and by synthetic methods. In the former method the physiological activity and iodine content of the dried thyroid tissue, the globulin therefrom and various products of hydrolysis from this globulin were determined quantitatively. In the second method two iodized amino-acid derivatives, not previously tested by quantitative methods, were prepared synthetically and their physiological activity studied quantitatively.

In thus tracing the active complex a number of important assumptions were made. First, that the activity of unaltered thyroid tissue depends quantitatively on its iodine content. Second, that the best method known for measuring this activity directly and quantitatively is the Reid Hunt acetonitrile test¹. Third, that in case the iodine is present in the products of hydrolysis in the same combination as in the globulin then, per unit of iodine, these will still possess an activity comparable with the original globulin. Fourth, that in case the iodine complex is an iodized amino-acid and that in case this is decomposed in the process of hydrolysis then the synthetic preparation of various iodized amino-acids or derivatives thereof and the quantitative testing of

¹ This *Journal*, 1, p 33, 1905

these per unit of iodine may determine the probable nature of the iodine complex. In other words, the actual quantitative physiological activity per unit of iodine as measured by the Reid Hunt method was taken as the crucial test for the presence or absence of the unaltered iodine complex.

The historical development of the relation of thyroid activity to iodine content need not be considered at this time, especially in view of the thorough reviews and extensive confirmatory experiments made by Reid Hunt and A. Seidell,² as well as the comparative histological and chemical studies by Marine in cooperation with Lenhardt and Williams.³ A careful study of these papers justifies the first assumption. The second assumption is also well taken provided the proper precautions are observed as shown by Reid Hunt and A. Seidell.⁴ Other methods for testing the physiological activity of thyroid substance, based on changes in blood pressure,⁵ on increasing the irritability of the depressor nerve,⁶ on changes in nitrogen metabolism⁷ and on curative effects in cretinism⁸ have been employed, but are not applicable in a quantitative study, nor are they as specific reactions.

Of the third and fourth assumptions we had no definite proof. The studies of Oswald⁹ and others show that during hydrolysis of thyreoglobulin only 30 per cent or less of the iodine remains in organic combination. The iodine thus combined is in the various fractions and qualitatively it has been determined¹⁰ that probably the greater activity remains in the more complex products.

² Bulletins 47 (1903) and 69 (1910) of the Hygienic Laboratory, U. S. Public Health and Marine Hospital Service.

³ *Johns Hopkins Hospital Bull.*, xviii, p. 359, 1907, *Journ. Inf. Dis.*, iv, p. 417, 1907, *Archives of Internal Med.*, i, p. 349, 1908, *Ibid.*, iii, p. 66, 1909, *ibid.*, iv, p. 440, 1909, *ibid.*, vii, p. 506, 1911, *ibid.*, viii, p. 265, 1911, *Journ. of Exp. Med.*, xiii, p. 455, 1911.

⁴ *Loc. cit.*, *Journ. of Pharmacol. and Exp. Ther.*, ii, p. 15, 1910.

⁵ von Fürth and Schwarz, *Pflüger's Archiv*, cxxiv, p. 113, 1908.

⁶ von Cyon and Oswald, *Pflüger's Archiv*, lxxxiii, p. 199, 1901, Asher and Flack, *Zeitschr. f. Biol.*, lv, p. 83, 1910.

⁷ Baumann, *Zeitschr. f. physiol. Chem.*, xxi, p. 487, 1896, *ibid.*, xxii, p. 1, 1896, *Münch. med. Wochenschr.*, xl, 1896.

⁸ E. Pick and F. Pineles, *Zeitschr. f. exp. Path. u. Ther.*, vii, p. 518, 1909-10.

⁹ *Arch. f. exp. Path. u. Pharm.*, lx, p. 115, 1908.

¹⁰ Pick and Pineles, *loc. cit.*

of hydrolysis where also the greater part of the organically combined iodine is found. What relation the activity bears to the iodine content therein has however not been determined. As stated above we have evidence that some of the iodine is split off as iodide, but we have no direct evidence that all the organically combined iodine found in the products of hydrolysis is still in the same complex or in the same structural relationship as in the original thyreoglobulin. A number of iodized amino-acids have been studied qualitatively as to physiological activity. In no case has thyroid activity been detected. The most conclusive results as to the inactivity of 3,5-iodo-*laevo*-tyrosine are those reported by Strouse and Voegtlin¹¹. Other observations on the inactivity of various iodized proteins, which on hydrolysis yield 3,5-iodo-tyrosine, also bear out these conclusions. The studies on other iodized amino-acids do not lead to definite conclusions. Thus von Furth and Schwarz¹² prepared and studied what they considered iodized phenylalanine, histidine and tryptophane. They reported all these substances as physiologically inactive, but gave no data indicating that they had really separated iodo-derivatives of these substances. Pauly¹³ however actually separated pure tetra-iodohistidine anhydride and tri-iodo-imidazol and reported that these substances increased the respiratory and pulse frequencies, although uniodized imidazol had no such action. These considerations lead us to conclude that for the present the validity of the third and fourth assumptions is unknown to us and that the true answers thereto are part of the problem in hand.

EXPERIMENTAL PART

The mode of attack has already been outlined above. The details as to the methods employed and the preparation of the substances studied are given below.

A Preparations

Dried hog thyroids Hog thyroids¹⁴ were freed mechanically from fat as much as possible and dried on glass plates in a current of air at 30-35°C

¹¹ *Journ of Pharm and Exp Ther*, 1, p 123, 1909

¹² *Pflüger's Archiv*, cxliiv, p 113, 1903

¹³ *Ber d deutsch chem Gesellsch*, xliii, p 2243, 1910

¹⁴ The raw material for this research was supplied by the Armour Laboratory Department

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The mass was then ground to a coarse powder and fat removed by ether in the cold. The remaining dry mass was then finely powdered. Duplicate determinations on this gave 0.243 and 0.250 per cent iodine.

Thyreoglobulin This was prepared as previously described¹⁵. Duplicate determinations on this gave 0.462 and 0.468 per cent iodine.

Iodothyryl (a) was prepared by the usual Baumann process from the above thyreoglobulin. The extraction with 95 per cent alcohol of the melanoidin precipitate was made in a continuous hot extractor. Duplicate determinations gave 5.81 and 5.85 per cent iodine.

Iodothyryl (b) was obtained in the same way from the melanoidin precipitate which separated in the complete hydrolysis of some of the same thyreoglobulin by 30-35 per cent sulphuric acid. This on analysis gave 7.51 per cent iodine.

Iodothyryl (c) was obtained from 40 grams of the same globulin by hydrolysis for three days at room temperature and for twenty-four hours at boiling temperature with 20-25 per cent phosphoric acid. Phosphoric acid was used as it was thought that possibly the oxidative action of sulphuric acid might have an injurious effect. This amount of globulin yielded 3.30 grams of melanoidin, containing 1.75 per cent iodine. The iodothyryl extracted from this represented 24 per cent of the weight and contained 4.44-4.46 per cent iodine. Thus only 61 per cent of the iodine in the melanoidin fraction was recovered in the alcohol extract.

Metaprotein (A₄) The filtrate from the melanoidin fraction above was neutralized with NaOH and the metaprotein separated and dried over sulphuric acid in a vacuum desiccator. This weighed 1.62 grams and contained 1.51-1.53 per cent iodine.

Primary albumose (A₅) The filtrate from above was half saturated with zinc sulphate after slightly acidifying with sulphuric acid. The precipitate obtained was dialyzed until free from sulphate. In this fraction there were recovered 3.1 grams containing 0.22-0.225 per cent iodine.

Secondary albumose (A₆) Obtained from the filtrate from above by complete saturation with zinc sulphate. The precipitate after dialyzing as above yielded 4 grams dry substance containing 0.069 per cent iodine.

The table (I) below gives a summary of the distribution of iodine in the different fractions above.

TABLE I

	WEIGHT RECOVERED	PER CENT OF IODINE THEREIN	WEIGHT OF IODINE	PER CENT OF TOTAL IODINE IN THE GLOBULIN
Melanoidin precipitate	3.30	1.74	0.0575	30.9
Metaprotein	1.62	1.52	0.0246	13.2
Primary albumose	3.01	0.22	0.0066	3.5
Secondary albumose	4.0	0.0695	0.0027	1.5
Undetermined iodine				50.9

¹⁵ This Journal, ix, p. 121, 1911

Phosphotungstic acid precipitate Another 40 grams of thyroglobulin were boiled with 25 per cent phosphoric acid for ninety-three hours. The filtrate from the melanoidin precipitate and metaprotein, after removal of the phosphoric acid by $\text{Ba}(\text{OH})_2$ and the excess of barium by sulphuric acid, was concentrated under diminished pressure to about 250 cc. This was then freed from proteose and peptone by the Kutscher tannin method¹⁶. The filtrate finally obtained here after removal of the excess of lead was boiled with BaCO_3 to remove the ammonia. The dissolved barium was again removed by sulphuric acid. The filtrate after acidifying with H_2SO_4 to 5 per cent strength was precipitated with phosphotungstic acid in the usual way. The precipitate after thorough washing with 2.5 per cent phosphotungstic acid solution was freed from phosphotungstic acid, barium and sulphate in the usual way. Duplicate determinations on the dry amino-acid mixture gave 0.0107 per cent and 0.0093 per cent iodine.

Another phosphotungstic acid precipitate from a hydrolysis by H_2SO_4 was worked up in the same way. This dry residue contained 0.0068 per cent iodine. The two samples were mixed and designated as P.T.A. Ppt. 1. This mixture contained 0.0073 per cent iodine.

Phosphotungstic acid filtrate (1) This was freed from phosphotungstic acid in the usual way. The amino-acid solution was evaporated to dryness. Duplicate determinations on the dry amino-acid mixture gave 0.0024 per cent iodine.

Phosphotungstic acid precipitate (2) This was obtained in the same way as the above from the partial hydrolysis by 10 per cent sulphuric acid of 141.6 grams of thyroglobulin containing 0.511 per cent iodine. The purified dry residue by analysis contained 0.0043 per cent iodine.

Phosphotungstic acid filtrate (2) The filtrate from the above was treated in the usual way. The dry purified amino-acid mixture left gave in duplicate determinations 0.0045 and 0.0043 per cent iodine.

Tetra-iodohistidine anhydride Histidine was prepared from ox erythrocytes by the method of Frankel¹⁷. Various methods were employed in trying to iodize the dichloride or the base itself but in no case were there indications of true absorption of iodine, but rather decomposition of the histidine. While this work was under way Pauly¹⁸ published his observations with the same conclusions as to the difficulty or inability to iodize histidine directly. At the same time, as stated above, he published his observations on tetra-iodohistidine anhydride. Following the methods given by Pauly¹⁹ the preparation of the methyl ester of histidine dichloride was carried out and from this the histidine anhydride by the Pauly modification²⁰ of the Fischer and Zuzuki method. The histidine anhydride was recrystallized from hot water a number of times to obtain the more

¹⁶ *Zentralbl. f. Physiol.*, xiv, p. 504, 1905

¹⁷ *Monatsh. f. Chem.*, xiv, p. 230, 1903

¹⁸ *Ber. d. deutsch. chem. Gesellsch.*, xlii, p. 2243, 1910

¹⁹ *Zeitschr. f. physiol. Chem.*, lxi, p. 75, 1910

²⁰ *Loc. cit.*

readily soluble laevorotatory form. This was then iodized according to the Pauly method. One determination on the snow-white product gave 63 per cent iodine (theoretical 65 per cent). The slightly lower value may be due to an admixture of a small amount of di-iodohistidine anhydride.

Iodized tryptophane Tryptophane was prepared from commercial casein by the Hopkins-Cole method.²¹ Several attempts were made to iodize the pure crystals by the method of Neuberg,²² but in no case was a substance obtained containing more than 6.3 per cent iodine. The preparation finally made for physiological testing was obtained by dissolving one milligram molecule of tryptophane in 4 cc. of $\frac{N}{2}$ NaOH, cooling by immersing in ice water and, while keeping cool and stirring well, adding drop by drop 6 cc. of aqueous N iodine solution. The mixture was allowed to stand at ice box temperature for twenty-four hours, then filtered off. The precipitate was well washed with cold water and dried over sulphuric acid in a vacuum desiccator. The product obtained is light brown in color, readily soluble in alkalis, reprecipitated on acidifying and liberates a very small amount of iodine to chloroform on shaking therewith. Duplicate determinations on this gave 41.5 and 41.9 per cent iodine (the theoretical for mono- and di-iodo-tryptophane are 38.4 per cent and 55.7 per cent respectively).

B Methods

Determination of iodine The Hunter²³ method with slight modifications was employed. The material to be analyzed, taken in quantities of 0.05-2 grams was mixed with 15 grams of fusion mixture and covered with 10 grams of fusion mixture as suggested by Hunter. To conduct the fusion the Roger's ring burner was found to be much more satisfactory in ensuring a uniform rapid heating without overheating. With the size of the flame once determined one finds ten minutes to be ample time to give a satisfactory, easily removable fusion. In the treatment with alkaline hypochlorite it was considered best to warm to 40°C for ten minutes. In acidifying it is very important to make sufficiently acid and then always to the same degree. Sulphuric acid of 25 per cent strength was used here and since the same amounts of fusion mixtures and hypochlorite were used in each case the acidity was well controlled by always adding the same amount of acid. In removing the excess of chlorine gentle boiling was continued for forty minutes after the negative test of the vapors by starch iodine paper. In this way the blank test on the reagents never was more than 0.1 cc. of a $\frac{N}{250}$ $Na_2S_2O_3 \cdot 5H_2O$ solution.

Physiological testing by the Hunt method The method employed was that of feeding the same quantity of iodine, in the different combinations, to white mice in such a manner as to make as certain as possible the entire consumption of the material fed. In order to do this each mouse was first

²¹ *Journ of Physiol*, LVII, p. 418, 1901

²² *Biochem Zeitschr*, VI, p. 276, 1907

²³ *This Journal*, VII, p. 321, 1910

fed for three or four days with cracker dust made into pellets of known weight. At the close of this preliminary feeding the unconsumed material was weighed and from this the average amount eaten per day determined. For ten days following this period each mouse then received this weight of cracker dust, with the incorporated iodine-containing substance, in the form of pellets. The control mice were fed in the same way with plain cracker dust pellets. At the end of the 10-day feeding period the acetonitrile was injected subcutaneously. Each dose administered in series I, II and III was contained in 1 cc. of fluid, in series IV-IX, in 0.5 cc., in series X, in 0.66 cc. In most cases the animals consumed the food very well. All the mice used were raised in the laboratory building on a diet of milk and crackers with occasional bits of lettuce until used for the experiment. Care was taken to compare mice of as nearly the same age as possible. In the tables below the litter number of each mouse is given. The ages of the mice of the various litters were as follows: Litter 2, 119 days, litter 3, 102 days, litter 4, 100 days, litter 5, 80 days, litters 6 and 10, 99 and 113 days respectively, litter 9, 115 days, litters 11-12, 125 and 135 days respectively, litters 13-14, 144 and 151 days respectively, litter 28, 85 days, litters 29-30, 59 and 66 days respectively, litters 31, 32 and 34, 95, 85 and 97 days respectively, litters 33-35, 101 and 89 days respectively, litters 36-37, 89 days, litter 38, 79 days, litters 56-60, 91-103 days.

C Discussion of the physiological tests

Thyreoglobulin Series I shows that thyreoglobulin possesses the full activity per unit of iodine when compared with the dried thyroid from which it was prepared. This is also confirmed by series IV where a decomposition product obtained from the globulin still shows the complete activity per unit of iodine. The whole of the physiological activity of the gland is therefore quantitatively in the thyreoglobulin.

Metaprotein As stated above, this still shows the full activity per unit of iodine although the percentage concentration of iodine has increased from 0.465 per cent in the thyreoglobulin to 1.52 per cent in the metaprotein.

Iodothyryn None of the iodothyryn preparations tested was found to bring about a resistance to acetonitrile more than three-fourths of that produced by the thyroid-tissue fed mice. The indications are that these preparations are all about equally inactive. Iodothyryn is therefore less active per unit of iodine than the thyreoglobulin. See series III and V.

Primary albumose This is still very active, as shown by series IV and VII, although the full activity per unit of iodine is not

shown to be present in every case tested. In this connection it may be mentioned that the results in series VI are of no value. This series VI, however, is an illustration of irregular results, due in all probability to impure acetonitrile. The acetonitrile was taken from a freshly opened bottle and found to smell decidedly of hydrocyanic acid. Before using, it was shaken twice with saturated potassium carbonate solution, dehydrated with P_2O_5 and twice distilled from fresh P_2O_5 . Finally it was redistilled and the fraction collected between 79 and 83°C. This distillate was used in series VI. For the later series this distillate was again purified in the same way three times and finally redistilled twice without the addition of P_2O_5 . Here the distillate was collected between 80.5 and 81.5°C.

Secondary albumose. This is much less active per unit of iodine than either the iodothyron preparations or the primary proteoses. Series VII* shows this, where the maximum dose resisted is only 40 per cent of the maximum dose resisted by the thyroid-tissue fed mice.

Amino-acids from the phosphotungstic acid precipitate and the phosphotungstic acid filtrate respectively. The results in series IX indicate that the former possess very little physiological activity as measured by the Hunt method. On the whole, however, the results here are very unsatisfactory as the mice did not eat the amino-acid mixtures well, there being two or more days' feeding left. The results indicate that these amino-acid fractions contain very little thyroid activity. This is better shown in series X where only one-tenth the quantity of iodine-containing substances was fed. Although the mice fed with dried thyroid tissue resisted an amount over two and a half times that of the control mice, still the mice fed with the same amount of iodine, but in the form of amino-acids, resisted very little, if any, more of the acetonitrile than the control mice. In other words, these amino-acid fractions show a very slight physiological activity, if indeed they possess any activity whatever.

Tetra-iodohistidine anhydride and iodotryptophane. These substances when fed in amounts representing ten times the amount of iodine fed as thyroid tissue do not appreciably increase the resistance to acetonitrile. See series II and VIII.

Table II gives a summary of the results above. The relative physiological activity is expressed (on the basis of feeding the same amount of iodine in each case) as follows: representing in each case, by 100, the largest dose of acetonitrile from which the thyroid-tissue fed mice recovered, then the other figures represent the proportions the limiting doses of the otherwise fed mice bear thereto.

TABLE II

	RELATIVE ACTIVITY	IODINE IN THE SUBSTANCE	TOTAL IODINE IN THE TISSUE
		<i>per cent</i>	<i>per cent</i>
Thyroid tissue	100	0.247	100.0
Thyreoglobulin	100	0.465	100.0
Metaprotein	100	1.520	13.2
Iodothyron	50-75	4.46-7.51	18.3
Primary albumose	80-100	0.220	3.5
Secondary albumose	40	0.0695	1.5
Amino-acids precipitated by phosphotungstic acid	0(+?)	0.0043	
Amino-acids not precipitated by phosphotungstic acid	0(+?)	0.0044	
Tetra-iodohistidine anhydride	0	65.00	
Iodotryptophane	0	41.70	

These results show that both the thyroid activity and iodine may be concentrated from thyroid tissue in the thyreoglobulin as well as in the metaprotein and iodothyron from the latter. Per unit of iodine, however, we have full activity retained in the thyreoglobulin and metaprotein only. In the primary albumose fraction we have a lowering in the percentage concentration of iodine and also a slight lowering in the physiological activity per unit of iodine. In the secondary albumose this is still more marked. In the amino-acid fractions the activity is extremely low if present. In view of the researches of Hunt and Seidell with various iodine compounds and in view of the results obtained here, we cannot attribute the protective action in any of these cases to iodine itself, but to a specific iodine-containing complex in the thyreoglobulin. It is significant to note that the highest physiological activity per unit of iodine is found in the original protein and in the more complex products of hydrolysis. Since the lowest products of hydrolysis are still less active per unit of iodine than the secondary albu-

mose it indicates either that the iodine group is altered in the hydrolysis, or that the iodine-containing group when in simpler combination or when separated, does not possess the full specific thyroid activity. That the iodine-containing group when once separated would not possess the full activity is not at all unlikely, but we would be inclined to expect it to show some activity, at least when given in amounts such as were employed by Strouse and Voegtlin with iodotyrosine and by the author in the experiments with tetra-iodohistidine anhydride and iodotryptophane. The indications as to the presence of tyrosine and tryptophane in iodothyron are very favorable, both from the chemical studies on iodothyron and also from similar studies on iodine-free melanoidins.²¹ It is not likely that the iodine is split off and then later added to the melanoidin fraction, it is more likely that it is already present in the globulin in the melanoidin-forming groups and remains in the original position in these groups, but that the groups themselves are changed in regard to each other and thus the activity affected to some extent, a poly-iodo derivative may be changed to a mono-iodo derivative and then may show decided differences in physiological activities. If this were not the case we would expect artificially iodized melanoidins to show a decided thyroid activity. Furthermore, it is not likely that sufficient hydriodic acid is split off in the early stages of the hydrolysis to yield as much iodine as is contained in the melanoidin fraction. Finally, it is not at all improbable that we here have to do with a specific iodophore group just as in hemoglobin we have the chromophore group containing the iron. The negative results with artificially iodized proteins speak strongly in favor of this view.

CONCLUSIONS

1 The full activity of thyroid tissue is contained in the thyreoglobulin fraction when this activity is measured by the Hunt method

2 The full activity per iodine unit is still present in the metaprotein fraction from this globulin, although the iodine content in the metaprotein fraction has been increased over threefold that of the globulin itself

²¹ Samuely *Hofmeister's Beiträge*, 11, p 355, 1902

3 The other products of the hydrolysis studied, primary albumose, iodothyrim and secondary albumose, show a gradual decrease in activity per unit of iodine in the order given

4 The amino-acid fractions precipitated and not precipitated by phosphotungstic acid from the partially hydrolyzed thyroglobulin still contain very small amounts of iodine and per unit of iodine are either extremely low in activity or entirely inactive

5 Tetra-iodohistidine anhydride and iodotryptophane do not possess thyroid activity as determined by the Hunt method

I wish to express my thanks to Prof A P Mathews for suggestions made in the course of the work

SERIES V—Continued

MOUSE	LITTER NO	FED DAILY WITH CRACKER DUST PLUS	FATAL DOSE OF ACETO-NITRILE	DEATH OCCURRED AFTER	DOSE OF ACETO NITRILE FROM WHICH RECOVERY OCCURRED
			mg per gm	hrs	mg per gm
(g) ♀	13-14	{ 0.0556 mg iodothy- rin (c) (=0.00247 mg I)	died while feeding		
(h) ♀	13-14		2.5	3½	

SERIES VI November 24–December 4

(a) ♀	29-30	{ 1 mg dried hog thy- roids (=0.00247 mg I)	2.5	< 24	
(b) ♂	29-30		3.0	1½	
(c) ♂	29-30		2.0	< 18	
(d) ♂	29-30	{ 1.123 mg primary albumose (A ₅) (=0.00247 mg I)	died while feeding		
(e) ♀	28		2.0	36-40	
(f) ♂	29-30				2.5
(g) ♂	28	{ 3.55 mg secondary albumose (A ₆) (=0.00247 mg I)	1.5	< 4	
(h) ♀	28		2.0	24-36	
(i) ♂	28		1.25	20-36	

SERIES VII January 13-23

(a) ♀	31-34	{ 1 mg dried hog thy- roid (=0.00247 mg I)	died while feeding		
(b) ♂	31-34				2.0
(c) ♀	31-34				2.5
(d) ♂	31-34				3.0
(e) ♂	31-34	{ 1.123 mg primary albumose (A ₅) (=0.00247 mg I)			2.5
(f) ♀	31-34				2.0
(g) ♀	31-34		2.8	> 6	
(h) ♀	31-34		3.0	> 8	
(i) ♂	31-34	{ 3.55 mg secondary albumose (A ₆) (=0.00247 mg I)	2.0	2½	
(j) ♂	31-34		1.2	> 4	
(k) ♀	31-34		1.0	24	
(l) ♀	31-34				1.2

SERIES VIII February 17-27

MOUSE	LITTER NO	FED DAIRY WITH CRACKER DUST PLUS	FATAL DOSE OF ACETO-NITRILE	DEATH OCCURRED AFTER	DOSE OF ACETO NITRILE FROM WHICH RECOVERY OCCURRED
			mg per gm	hrs	mg per gm
(a) ♀	33-35		0 55	<18	0 45
(b) ♀	33-35				0 40
(c) ♀	33-35				0 35
(d) ♀	33-35				
(e) ♂	33-35	1 mg dried hog thy-roids (=0 00247 mg I)	4 0	2	2 0
(f) ♂	36-37		died while feeding		
(g) ♀	36-37		3 0	6	
(h) ♀	36-37				
(i) ♂	33-35	0 0059 mg iodotryp- tophane (=0 00247 mg I)	0 55	>36	
(j) ♂	33-35		1 6	2½	
(k) ♀	36-37		1 0	18	
(l) ♀	36-37		0 45	>24	
(m) ♂	33-35	0 059 mg iodotryp- tophane (=0 0247 mg I)	1 0	< 3	0 5
(n) ♂	33-35				
(o) ♀	33-35		0 70	<18	

SERIES IX March 12-22

(a) a Ser VIII	33-35	1 mg dried hog thy- roid (=0 00247 mg I)	4 0	< 6	3 5
(b) b Ser VIII	33-35		did not eat, not	injected	3 0
(c) c Ser VIII	33-35				
(d) n Ser VIII	33-35				
(e) ♀	38	33 6 mg P T A Ppt 1 (=0 00247 mg I)	died while feeding		0 8*
(f) ♀	38		died while feeding		
(g) ♂	38				
(h) ♂	38	100 mg P T A Filt 1 (=0 0024 mg I)	1 0	< 3*	
(i) ♂	38		0 8	<18*	
(j) ♂	38		0 6	<18*	

* Two or more days feeding left. This experiment is not reliable as animals were used which had recovered in previous experiments and the differences in age were too great for such young animals.

SERIES X

MOUSE	LITTER NO	FED DAILY WITH CRACKER DUST PLUS	FATAL DOSE OF ACETO- NITRILE	DEATH OCCURRED AFTER	DOSE OF ACETO- NITRILE FROM WHICH RECOVERY OCCURRED
			mg per gm	hrs	mg per gm
(a) ♂	56-60		0.5	<10	
(b) ♂	56-60		died while	feeding	
(c) ♀	56-60				0.4
(d) ♂	56-60				0.35
(c) ♂	56-60	0.1 mg dried hog thyroid (=0.000247 mg I)	1.2	3½	
(f) ♂	56-60				1.1
(g) ♀	56-60		not injected, gravid		1.0
(h) ♀	56-60				
(i) ♂	56-60	5.74 mg P T A Ppt 2 (=0.000247 mg I)	0.5	48*	
(j) ♂	56-60		1.0	<6†	
(k) ♂	56-60		0.8	<16	
(l) ♀	56-60				0.4
(m) ♂	56-60	5.61 mg P T A Filt 2 (=0.000247 mg I)	1.0	3½	
(n) ♂	56-60		0.8	<12	
(o) ♂	56-60		0.7	24	
(p) ♂	56-60				0.5

* Two days feeding left

† About one day's feeding left

EXPERIMENTS BEARING ON THE FUNCTIONS OF THE LIVER IN THE METABOLISM OF FATS I

By H S RAPER

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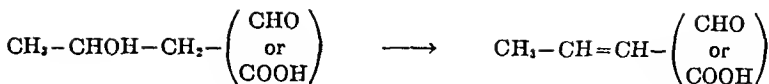
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Since the publication of Knoop's experiments on the oxidation of phenyl substituted fatty acids in the animal body, a large amount of work has been directed to the object of determining the exact nature of the intermediate reactions involved in the breakdown of fatty acids to the ultimate end products, carbon dioxide and water. It would occupy too much space to give a complete summary of this work and would, further, be needless, since an excellent account of the work has recently been published elsewhere¹. It is sufficient to say that most of the results, so far obtained, support the hypothesis that in the catabolism of fatty acids the long chain of carbon atoms is gradually broken down by a successive series of β -oxidations involving the removal of two carbon atoms at a time. The first oxidation product is a β -ketonic acid or possibly a β -hydroxy acid, though much of the evidence points to the conclusion that β -hydroxy acids are produced by reduction of the ketonic acids. In some instances, the formation of α,β -unsaturated acids has been observed, but whether they arise by direct oxidation or indirectly, by the removal of the elements of water from a β -hydroxy acid, is not yet decided. Most of these exceedingly interesting results have been obtained by observations on the fate of phenyl or furfuryl derivatives of the lower fatty acids and it has been assumed that the same series of changes is the one which represents the mode of oxidation of the higher fatty acids when they are utilized as a source of energy in the animal body. This assumption receives support from the fact, established by Embden and his co-workers, that on perfusion of a surviving liver with blood containing caproic, octoic or decolic acids,

¹ Dakin *Oxidations and Reductions in the Animal Body*, Longmans, Green and Company, 1912

aceto-acetic acid was produced, this being inferred from the increased amount of acetone obtained from the blood at the end of the experiment, whereas acids containing an odd number of carbon atoms gave no aceto-acetic acid. The excretion of the "acetone bodies" in the urine in human or experimental diabetes may also be accepted as evidence in favor of the above assumption, since in these conditions it is beyond doubt that fat is being used up in relatively large quantities as a source of energy. The fact that in such conditions, and especially in the experimental ones, the liver often contains a larger amount of fat than normal, has been taken to indicate that it is to this organ that the fat first comes when it is to be oxidized, since the other active organs of the body do not exhibit this fatty infiltration to anything like the same extent. When, however, we come to examine the nature of the fat in the normal liver, it is found to contain a much larger amount of unsaturated fats than that in the fat depots or, more correctly, the fat of the liver taken as a whole is more unsaturated than the connective tissue fat. This is also true of the other active organs, such as the kidney and heart, but the difference is not quite so marked. The exact meaning of the presence of these unsaturated acids is not yet clear. Does the presence of these highly unsaturated acids indicate that they represent a stage in the normal breakdown of fat in the liver, or does it represent a stage in the synthesis of fat or are the acids essential constituents of the protoplasm of the cell which enable it to carry out functions not directly associated with the catabolism of these acids themselves?

With the knowledge at present at our disposal, it is not possible directly to answer any of these propositions, and it was with the object of trying to clear away some of the difficulties that the present work was started. We know so little about the synthesis of fats in the liver that the second possibility just mentioned cannot be put to the proof. If, however, we accept the view of Leathes, that fatty acids are synthesized by the condensation of acetaldehyde, with coincident oxidation and reduction at the appropriate stage, then it is easy to understand how unsaturated acids could arise in such a process by removal of the elements of water from the hydroxy aldehydes or acids formed, just as β -hydroxybutyric aldehyde or acid loses water and is converted into crotonic aldehyde or acid as the case may be.



Some interesting experiments on the formation and distribution of the unsaturated acids in the liver have been carried out by Leathes and his co-workers² It has been shown that the fatty acids, contained in the lipoid substances of liver, are as a rule more unsaturated than those present as glycerides, so that it seems probable that some of the unsaturated acids are destined to be built up into phosphatides and it is possible that the liver may be specially concerned in their manufacture In a further series of experiments, in which animals (rats and cats) were fed with oils having a high iodine value, it was found on examination of the fat from the various organs that the liver fatty acids had a higher iodine value than that of the oil administered, no such change being observed in the fat from the other organs The amount of fat, too, in the liver, appeared to be larger than normal in some cases and this also was true to some extent of the spleen From these experiments it was concluded that "the liver takes up fat conveyed to it by the blood and changes the fatty acids in such a way as to increase their power of absorbing iodine This may be interpreted as due either to the introduction of new unsaturated linkages, or to the transposition of existing ones from situations in which they are less liable to saturation by halogens to others in which they are more so" Another possibility may be added, namely, that the liver has some selective affinity for the highly unsaturated acids and takes them up in preference to the more saturated ones

The work described in the present paper was undertaken with the object of determining definitely, if possible, whether saturated higher fatty acids, when taken up and metabolized in the liver, became unsaturated, as it was thought that this would tell us whether the phenomena observed by Leathes and Meyer-Wedell were due to a selective affinity of the liver for unsaturated acids In order to accomplish this, it was necessary to have carried to the liver some saturated acid that could be separated subsequently from the acids normally present in the liver and examined with respect to its power of combining with iodine

In view of Friedmann's results³ on the fate of methylated amino-acids in the organism, which showed that the methyl-amino group in these acids apparently protected them from complete oxidation, it was decided to use phenylamino-stearic acid, $C_{18}H_{31}-CH(NHC_6H_5)COOH$, in the hope that, although the α -carbon atom might be immune from attack, the long chain of carbon atoms might still be open to oxidation. Moreover, phenylamino-stearic acid is insoluble in petroleum ether and can therefore be easily separated from the fatty acids normally present in the liver. The acid, however, was not easily absorbed, so this substance had to be rejected. Further, the small amount of acid absorbed was apparently oxidized for none could be found in the liver or connective tissues. This was confirmed by examining the urine for *p*-amino-phenol. After making the urine alkaline with sodium carbonate and extracting with ether, a residue was obtained from the ether which gave a very strong indophenol reaction. Enough was not obtained for further identification. A similar result was obtained when α -phenylamino-hexanoic acid was administered. In this case 4 grams of the acid given to a cat of 3.7 kilos proved fatal. The phenylamino group in these acids is therefore not immune from attack.

The next experiments were of a different nature. Higher fatty acids substituted with bromine in the α -position were administered and an attempt was made to separate the bromo-acids from the tissue or organ fatty acids by converting the former into phenylamino acids—a change which is easily brought about by heating them with aniline. The free acid, however, even when mixed with meat, usually caused vomiting in cats, so this method of experiment was abandoned. In one case, in which a cat absorbed 7 grams of the acid in eighteen hours, none could be detected in the liver or connective tissues by means of aniline.

The next method chosen was the administration of coconut oil of which about 40 per cent of the fatty acids are volatile in steam, these acids moreover do not take up iodine. The method adopted was to separate the volatile acids by steam distillation from the liver of animals to which the oil had been administered and determine whether they had become unsaturated by estimating

³ Friedmann *Beitr z chem Physiol u Path*, xi, p 158

the iodine absorption. Since the small amount of higher fatty acids, separated from the fatty acids of a normal liver by steam distillation, take up iodine, the method adopted had to be a differential one. Some of the experiments indicated that the acids recovered in this way from the liver had acquired the property of absorbing iodine to a slight extent. The effect was not very marked and it does not appear to be sufficiently striking to prove the assumption that the liver desaturates these acids. It is possible, however, that only the higher fatty acids, with 16, 18 or 20 carbon atoms, undergo this action to a marked extent, especially if the acids produced have to be built up into phosphatides.

The oil was administered to the animals by three methods, namely, by the mouth, by introducing soap solutions into the small intestine and by intravenous injections of fine emulsions of the oil. In the course of the experiments, some interesting results were obtained which have a bearing on the selective functions exercised by the liver in the mobilization of fat. Since cocoanut oil contains about 40 per cent of acids, volatile in steam, it was possible to estimate, roughly, the total amount of oil in the various tissues, after administration of a given amount of cocoanut oil to an animal. Many factors influence the rate at which volatile acids distil in steam so the results are only roughly quantitative. Controls have shown however that they are accurate enough to enable one to interpret them in the way that has been done.

When oil is given, mixed with food, it is not absorbed very rapidly and, after short periods of five to twelve hours, about 6 per cent of the oil absorbed is found in the liver. In the connective tissue fat its presence is just detectable. By infusion of cocoanut oil soaps into the intestine about 30 per cent of the absorbed fatty acids is found in the liver. The condition of the intestine at the end of these experiments was very abnormal, so too much stress cannot be laid upon the results. By intravenous injection of fine emulsions of the oil, none of the administered oil could be detected in the connective tissues, but from 25 to 60 per cent was found in the liver. This remarkable selective activity exhibited by the liver in the taking up of fat from the circulating blood is probably due in part to the effect of the anaesthetic. It was found, for instance, that a dog, which had cocoanut oil given by the mouth and later half a grain of morphia followed by ether anaesthesia, had

twice as much of the oil in its liver as dogs similarly fed but receiving no morphia and ether. The amount of oil in the liver was however, even then, not so great as in a dog to which the oil was given intravenously. Another factor which probably has an influence on this selective action of the liver is the rate at which the oil enters the circulation. In the feeding experiments, the oil was absorbed by cats, rarely at a greater rate than 0.5 gram per hour, whereas in the intravenous injections it was given at the rate of 0.77 to 1.37 grams per hour.

Another factor which would explain in part the divergence between the results of the feeding experiments and the intravenous injections is the possibility that the lower fatty acids in cocoanut oil, for instance, capric and lauric acids, may be absorbed, not as glycerides but as sodium salts and may not display the same tendency to accumulate in the liver as the glycerides do. It is obvious, when we consider the absorption of the sodium salts of the whole series of fatty acids, the lower members of which are absorbed without further change and the higher members largely synthesized into glycerides before entering the circulation, that there must be a gradual transition from one method of absorption to the other. It is possible then that intermediate acids, such as capric and lauric, reach the circulation partly as sodium salts, when their glycerides are introduced into the intestine, and since these acids are among those which were used to localize the cocoanut oil in the tissues because of their volatility in steam, the amount of volatile acid obtained from any organ would only indicate the *minimum* amount of cocoanut oil in that organ, because the fat entering the organ might contain a smaller percentage of volatile acids as glycerides than the original cocoanut oil. This hypothesis was tested by administering cocoanut oil to a dog and collecting the lymph from the thoracic duct during absorption of the oil. It was found that the mean molecular weight of the acids present in the lymph as glycerides was much higher than that of the acids in the oil given and was still higher than that theoretically calculated allowing for the fat normally present in lymph. The divergence in results obtained in the feeding experiments and intravenous injections may therefore be partly due to the fact that some of the lower acids from the cocoanut oil enter the circulation as sodium salts and consequently do not accumulate in the liver.

One other possibility remains to be considered, namely, that the fat taken up by the liver, when fine emulsions were introduced intravenously, was retained mechanically and the phenomenon was therefore purely one of obstruction. None of the experiments disprove this conclusively, as an emulsion has not yet been obtained so fine that a small fraction of the fat given does not remain in the lungs or spleen. This occurs with emulsions so fine that the majority of the fat particles are less than 2μ in diameter and certainly not more than 4μ . The emulsions were injected into the external jugular vein so that all the fat had to pass the pulmonary capillaries before entering the systemic circulation. In the earlier experiments, in which the emulsions were not so fine as in the later, a considerable amount of the fat remained in the lungs, the lung capillaries thus acting as filters and retaining the larger particles. Of the fat which finally entered the systemic circulation, none was found in the connective tissues, the intestinal mucous membrane or the kidneys, whereas the liver contained 30 to 40 per cent. With the finest emulsions, which resembled chyle in microscopic appearance, less than 2 per cent of the oil given was retained by the lungs and less than 3 per cent by the spleen, whereas 25 per cent was in the liver. It seems probable, therefore, that this rapid fatty infiltration of the liver by the injected cocoanut oil is a phenomenon identical with that observed in conditions of lipaemia, such as, for instance, occurs in dogs after excision of the pancreas. There seems to be every indication, therefore, when fat is brought rapidly into the circulation, that it is taken up selectively by the liver. It is hoped to make further use of this selective function, in a manner similar to that described in the present series of experiments, in order to determine the changes which occur in fatty acids other than those of cocoanut oil when taken up by the liver.

EXPERIMENTAL PART

The estimation of total fatty acids in the liver, when carried out, was done by Liebermann's method of direct saponification, and all iodine values were determined by Wij's method.

For the separation and estimation of the volatile acids, the organ was heated with about its own weight of 50 per cent caustic potash, until complete solution was accomplished, then alcohol was

added and the heating continued until saponification of the fat was complete. The liquid was transferred to a large flask, acidified with sulphuric acid and distilled in a current of steam, 2.5 liters of distillate were collected in every case. The distillate was made alkaline with caustic potash in order to dissolve the fatty acids and then evaporated to small bulk on the water bath. This residue was transferred quantitatively to a Liebermann flask and the fatty acids soluble in petroleum ether estimated in the usual way. From a portion of the fatty acids obtained, the small amount of unsaponifiable matter usually present was removed by converting the acids into soaps in 60 per cent alcohol and extracting the solution with petroleum ether. The purified fatty acids separated from the soap solution by acidification and extraction with petroleum ether then served for the determination of the mean molecular weight.

For the volatile acids from the connective tissues, a fair sample of connective tissue from the subcutaneous tissues, the omentum and the subperitoneal fatty tissues was taken and, after saponification and liberation of the fatty acids, was distilled in steam, 2.5 liters of distillate being collected. A control, obtained by adding 1 gram of cocoanut oil to 80 grams of connective tissue fat and subsequent saponification and distillation in steam, showed that the actual weight of volatile acids obtained from the connective tissue fat gave very little quantitative indication of the amount of cocoanut oil present but the mean molecular weight of the volatile acids immediately showed that the presence of cocoanut oil in the connective tissues could be detected in this way.

For the experiments on cats, four control experiments were carried out. Three with the livers of normal cats without any addition, and one with a cat's liver to which a gram of the oil had been added before saponification. Two controls were also carried out with dog's liver on similar lines. The results are collected in table I.

It will be seen, on reference to this table, that from a normal cat's liver, on an average, 109 mgm of volatile acids soluble in petroleum ether were obtained, with a mean molecular weight of about 250, and these absorbed 61.4 mgm of iodine. When 1 gram of cocoanut oil was added to the liver and the subsequent treatment was the same, then the amount of volatile acids obtained

rose to 424 mgm. The iodine absorbed by these acids was 49.2 mgm. Similarly, with roughly the same amount of dog's liver, 123 mgm. of volatile acids were obtained with a molecular weight of 255.5 and absorbing 65.9 mgm. of iodine. When coconut oil was added to the same amount of the same liver, an increased yield of volatile acids was obtained with a corresponding decrease in their molecular weight and a slight diminution in the iodine absorption.

Feeding experiments

In the experiment on cats, the oil was usually mixed with minced lean meat or with boiled codfish which contain less than 1 per cent of fat. When the animal was killed, the contents of the alimentary tract were taken and the fat estimated, in order to gain some idea of the amount of oil absorbed. In the experiments with dogs, the oil itself was given by the mouth and was usually readily taken. As in the experiments with cats, the stomach and intestinal contents were worked up to find the amount of oil actually absorbed. The results are collected in tables II and III.

On reference to table II, it will be seen that more volatile acids are obtained from the liver than in the control cats. In three out of the four experiments, the iodine absorption of the volatile acids is higher than in the controls, the most marked result in this direction being shown by the cat which had been receiving oil for nine days. In this experiment the iodine absorption was 50 per cent above the control average. In spite of this, the desaturation of the volatile acids can only have taken place to a slight extent, as in the most marked case, with an iodine absorption of 92 mgm., it only accounts for a rise in the iodine value of the volatile acids from 0 to 6. In all four cases the mean molecular weight of the volatile acids from the connective tissues is below the control, showing that some of the oil had been retained there. The result is most marked, as would be expected, in the cat which has received the largest amount of oil. In the other three cases, in which the animal was killed within twelve hours of administration of the oil, if allowance be made for the amount of volatile acids from a normal liver, and assuming that the amount of volatile acids obtained is equivalent to two and a half times its weight of oil, we find that between 5 and 6 per cent of the oil absorbed was in the liver.

In the experiments with dogs (table III) the presence of coconut oil in the liver is also revealed by the amount of volatile acids obtained, but it is not quite as marked as in the experiments with cats. The case in which the most notable increase in volatile acids occurred is the one in which the dog was given morphia and ether subsequent to the administration of the oil. In only three, out of the five experiments, is the iodine absorption above the average for the controls, being highest in the dog which had been anaesthetized for four and one-half hours. Three grams of urethane and ether for only half an hour appears to produce little effect on the accumulation of oil in the liver.

Infusion of soap into the intestine

The object of these experiments was to get rapid absorption and with this the possibility of a greater accumulation of the oil in the liver. It was hoped thereby to get more satisfactory evidence as to the desaturation of the saturated volatile acids than was obtained from the feeding experiments. In these experiments, cats were used. Urethane was given, followed by ether, and cannulae were inserted into the intestine just below the pylorus and just above the ileo-caecal valve, the lower cannula being fixed to a burette. The solution to be administered was injected periodically through the upper cannula, and at the end of the experiment the intestinal contents were removed in order to determine the exact amount of fatty acid absorbed. It was found that whenever the fluid infused contained chiefly soaps, the intestine at the end of the experiment was very congested and the mucous membrane had partly desquamated so that the condition of the absorbing area had been very abnormal. On the other hand, when emulsions containing fatty acids or free hydrochloric acid were introduced, the intestine at the end of the experiment appeared quite normal and healthy. In spite of the numerous and varied mixtures introduced into the intestine, containing sometimes oil, sometimes free fatty acids or soaps, together with bile salts and glycerin, and although acid, neutral and alkaline fluids were tried, in only four of the experiments was enough absorption obtained to make it worth while searching for volatile acids in the liver. The results are collected in table IV.

The experiments show that the accumulation of the oil in the liver was much more marked than in the feeding experiments, the amount varying between 25 and 33 per cent of the fatty acid given. The iodine absorbed by the volatile acids was also higher than in the feeding experiments and still higher than in the controls. These experiments, then, indicate that desaturation of the volatile saturated acids from the oil has taken place, but, again, only to a slight extent.

Intravenous injection of emulsions

The object of these experiments, as also that of the previous set, was to introduce fat rapidly into the circulation with the hope that more would be taken up by the liver and in consequence give a better chance for the detection of desaturation if it occurred. In all the experiments performed, the amount of fat taken up by the liver was greater than in the feeding experiments, but only slight evidence of desaturation was obtained. In the earlier experiments, the emulsions were relatively coarse, and rather less than half the oil given was retained by the lungs. These emulsions were made by shaking the oil with a 0.15 per cent emulsion of lecithin in normal saline. The emulsions usually contained about 4 per cent of oil. The amount of oil was estimated before each experiment so that the amount introduced could be accurately determined. It was discovered later that much finer emulsions could be made by using caseinogen instead of lecithin as emulsifying agent. These emulsions were prepared as follows:

Four grams of casein were heated on the water bath in a porcelain dish with 25 cc of normal saline and 4 cc of decinormal sodium hydroxide until solution was practically complete, 8 grams of coconut oil were now added and the mixture rubbed up with a pestle until drops of oil were no longer visible. The dish was now removed from the water bath and the rubbing up continued until with gradual cooling the mass became gelatinous. If too much saline is used to dissolve the caseinogen the mass does not set when cold and the emulsion is not quite as fine as when the liquid sets to a jelly on cooling. In case the cold mixture is still creamy, it should be warmed up again until a little water has evaporated and the grinding continued until it is cold. An alkaline salt solution was now made containing 160 cc of normal saline and 1 cc of decinormal soda for each gram of oil used. The gelatinous mass was rubbed up in a mortar in small portions with a few cubic centimeters of the salt solution until dissolution was complete.

Finally the creamy solution thus obtained was diluted with the remainder of the salt solution, filtered through cotton wool and finally through filter paper. If the preparation of the emulsion has been successful, the whole of the fat should be in minute particles which show Brownian movement. No particles with a diameter greater than 4μ should be present and these should be few in number. Almost the whole of the fat is usually in particles less than 2μ in diameter. On standing in the cold, practically no separation takes place.

The method of administration was as follows

Urethane and ether were used as anaesthetics and, in the case of dogs, morphine and ether. A cannula was inserted into the external jugular vein and this was connected to the burette containing the emulsion so that the rate of entry of the fluid could be easily adjusted. The burette was surrounded by the outer tube of a Liebig's condenser through which water was allowed to circulate at a temperature of 40°C . About 100 cc of the emulsion was usually given in a couple of hours, the rate of administration of the oil being then about 2 grams per hour. The animal was kept alive for a further two to four hours, and then killed.

The results of these experiments are collected in table V. With the exception of the last experiment, the method of determining the amount of oil in the liver was the same as in the previous experiments, that is, by distillation in steam. In the last experiment, the amount of oil going to the liver was determined by excising a lobe of the liver previous to the injection of the emulsion and then comparing the amount of fat in this lobe with the amount in the rest of the liver at the end of the experiment. The result is approximately the same as obtained by the distillation method and serves to confirm the results obtained by it. In this case the spleen was examined for the presence of cocoanut oil by comparing the mean molecular weight of the acids from a normal spleen with that of the acids found in the spleen after the injection of the oil, the mean molecular weight of the acids in the oil being 212. In this case it was found that 0.09 gram of the oil was present in the spleen. It will be seen, from the tabulated results, that there is marked evidence that the fat accumulates in the liver, but again the phenomenon of desaturation is only slightly indicated, the mean iodine absorption of the volatile acids being 71.9 mgm as opposed to 61.4 mgm in the controls.

In conclusion, two experiments were performed on dogs in which, two hours after a meal of cocoanut oil, an attempt to collect the

lymph flowing from the thoracic duct was made. The object of these was to determine whether the fat in the chyle had the same composition as that administered, or, if of different composition, whether the difference was caused by a failure of all the volatile acids to undergo the glyceride synthesis. In one of the experiments, although 56 cc of lymph were collected, it contained so little fat in addition to what one might normally expect to be present in lymph, that no deductions in reference to the point in question could be made. In a second experiment only 7.5 cc of lymph were collected as the animal died owing to a misadventure with the anaesthetic. The lymph however contained 5.37 per cent of fat and was sufficient for the purpose of the experiment. On examination of the fatty acids obtained from the chyle fat, the following results were obtained. Mean molecular weight, 236, iodine value, 19.1. The iodine value of the oil given was 7.7 and the mean molecular weight of its fatty acids, 212. Assuming the iodine value of the fat normally present in lymph to be 90, and its molecular weight 284, which are approximately what one would expect, then if enough of the normal fat were present to raise the iodine value from 7.7 to 19.1 the mean molecular weight would only be 222, whereas it was found to be 236. It thus appears as if the lower fatty acids in cocoanut oil do not completely undergo the glyceride synthesis during absorption.

SUMMARY

1. Cocoanut oil administered to cats or dogs by the mouth can be detected in the liver in five or six hours. The amount present after times varying from five to twelve hours does not exceed 6 per cent of that absorbed.

2. If cats be anaesthetized (urethane and ether), and a solution of cocoanut oil soaps containing glycerin and bile salts be run into the small intestine, then about 30 per cent of the absorbed fatty acid is found in the liver.

3. When cocoanut oil is given to cats or dogs intravenously in the form of a very fine emulsion, containing about 4 per cent of the oil, then from 25 to 60 per cent of the oil which enters the systemic circulation is found in the liver.

4. It is probable that the greater retention of the oil by the liver, when it is administered in the form of soap or a fine emulsion, is

partly due to the anaesthetic and partly to the rapidity of administration

5 When coconut oil is being absorbed, the fat in the chyle contains fatty acids with an average higher molecular weight than those in the oil administered. It is probable, therefore, that the lower fatty acids in the oil are partly absorbed as sodium salts.

6 The volatile acids obtained from the liver in the above sets of experiments absorbed more iodine than the volatile acids from normal livers. The increase was not great but it probably indicates that saturated fatty acids containing 10, 12 or 14 carbon atoms may become unsaturated in the liver.

I wish to express my thanks to Dr V E Henderson for assistance in the thoracic duct experiments and to Professor Leathes for much valuable help and criticism.

TABLE I

TISSUE	LIVER			CONNECTIVE TISSUE	KIDNEY	
	Volatile acids	Iodine absorbed	Mean molecular weight	Mean molecular weight of volatile fatty acids	Volatile fatty acids	Mean molecular weight
	mgm	mgm			mgm	
Cat—Liver, 50 gms	100	65.7	251	259	591	233
Cat—Liver, 72 gms	120	56.2				
Cat—Liver, 90 gms	108	62.3				
Cat—Liver, 68 gms + 1 gm oil	424	49.2				
Cat—Connective tissue fat, 80 gms, + 1 gm oil				220		
Dog—Liver, 67 gms	115	71.5	254			
Dog—Liver, 70 gms	132	60.3	257			
Dog—Liver, 70 gms + 0.5 gm oil	(a)382	56.5	215			
	(b)400		216			

TABLE II
Experiments on Cats

AMOUNT OF OIL ABSORBED AND DURATION OF EXPERIMENT	LIVER				KIDNEY		SPLEEN		HEART		CONNECTIVE TISSUE
	Per cent of fatty acids	Iodine value	Molecu- lar weight	Volatile acids in milli grams	Iodine absorbed by vola- tile acids	Molecular weight of volatile acids	Per cent of fatty acids	Iodine value	Per cent of fatty acids	Iodine value	Molecular weight of volatile acids
102 gms oil, in 9 days	11.88	102.9	267	607	92.4	220	3.29	95.8	2.71	120.8	220
8 gms oil, in 12 hours	6.48	122.0	274	273	73.0	236	6.74	57.9	2.51		246
2 gms oil, in 5 hours	4.64	121.7		152	66.7	253					241
3.6 gms oil, in 8½ hours	4.98			180	58.0	251					242

TABLE III
Experiments on Dogs

	LIVER						OBSERVATIONS
	Fatty acids	Iodine value	Molecular weight	Volatiles acids from 0.8 gms liver	Iodine absorbed	Molecular weight	
	per cent			mgm	mgm		
8.45 kilos, 21 gms oil in 12 hours	2.65			149	77.5	235	
6.3 kilos, 3.4 gms oil in 6 hours	2.76	124	288	157	62	246	
8.2 kilos, 6.7 gms oil in 6 hours	2.95	118	283	182	77.2	244	
8.2 kilos, 19 gms oil in 7 hours	3.51	115.7	278	148	53.5	235	
6.5 kilos, 10.5 gms oil in 7 hours	3.92	129.9	288	142	69.2	253	Ether for $\frac{1}{2}$ hr in middle of absorption period 3 gms urethane
5 kilos, 12.8 gms oil in 7 hours	4.57	105.3	279	212	84.7	244	0.5 gram morphia Ether $4\frac{1}{2}$ hours

TABLE IV

COMPOSITION OF EMULSION	AMOUNT OF FATTY ACID ABSORBED	DURATION OF EXPERIMENT	LIVER				OBSERVATIONS
			Total fatty acids	Volatile acids	Iodine absorbed by volatile acids	Molecular weight of volatile acids	
Soaps and glycerin	grams 2 0	hours 4	per cent 5 70	mgm 367	78 7	218	Mucous membrane desquamated
Soaps, glycerin, little free fatty acid	1 9	4	6 23	303	95 2	235	Mucous membrane desquamated
Fatty acids, soaps glycerin, bile salts	1 2	4 25		245	75 2	233	Mucous membrane desquamated
Fatty acids, glycerin, bile salts	1 4	6 5		227	99 7		Mucous membrane normal

TABLE Va

ANIMAL	EMULSIFYING AGENT	OIL INJECTED	OIL RETAINED BY LUNGS	OIL ENTERING SYSTEMIC CIRCULATION	PERCENTAGE OF OIL ENTERING SYSTEMIC CIRCULATION WHICH WAS FOUND IN LIVER	DURATION OF EXPERIMENT
		grams	grams	grams		hours
Cat 1	Lecithin	6 0	2 57	3 43	60	4 0
Cat 2	Lecithin	4 65	1 56	3 09	43	5 3
Cat 3	Lecithin	4 35	2 13	2 22	38	5 2
Cat 4	Caseinogen	3 7	0 35	3 35	43	4 0
Cat 5	Caseinogen	3 42	0 07	3 35	25	5 5
Dog	Caseinogen	11 15	4 42	6 73		5 25

TABLE Vb

ANIMAL	LIVER					KIDNEY		INTESTINAL MUCOUS MEMBRANE		CONNECTIVE TISSUE FAT	SPLEEN
	Total fatty acids	Mean molecular weight of fatty acids	Total volatile acids	Iodine absorbed by volatile acids	Mean molecular weight of volatile acids	Total volatile acids	Mean molecular weight of volatile acids	Total volatile acids	Mean molecular weight of volatile acids	Mean molecular weight of volatile acids	Total fatty acids per cent and molecular weight
	per cent		mgm	mgm		mgm		mgm			
Cat 1	8.56	265	932	68.0	206	244	252	81	266	251	
Cat 2	8.80		643	73.5	212	241	243	172	265	257	
Cat 3	5.83		437	85.7	218			60	246	251	
Cat 4	4.87		685	61.0	204						3.63% *247
Cat 5	4.89										
Dog	3.39	269	384	71.5	219					253	

*Corresponds to the presence of about 90 mgm of coconut oil assuming normal spleen fatty acids to have a molecular weight of 284

THE INFLUENCE OF PHLORHIZIN ON DOGS WITH ECK'S FISTULA

By J E SWEET AND A I RINGER

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(Received for publication, January 30, 1913)

The liver plays a very important rôle in the carbohydrate metabolism. Its gluco- and glycogenetic functions have been the subject of a very thorough study for over half a century, and a great deal of light has been thrown on it. Its influence on the course of pancreatic and phlorhizin glucosuria has unfortunately been limited to but three series of experiments. Markuse¹ in 1894 showed that extirpation of the pancreas in frogs does not bring about any glucosuria if the liver is extirpated at the same time. A year later Montuori² published the results of his experiments in which he showed that no glucosuria followed the extirpation of the pancreas in dogs if the blood vessels leading to the liver were tied off. In 1907 Rosenfeld³ published experiments on dogs with Eck's fistula. He showed that the injection of phlorhizin brought about no glucosuria. These experiments led their respective authors to far-reaching conclusions. Rosenfeld, in fact, went so far as to assert that no glucosuria can arise except from glucose that has passed a glycogen stage. He differentiates between "transglycogenic" and "aglycogenic" glucose. By the former, he understands glucose that has passed through the liver and has been converted into glycogen. This sugar can give rise to glucosuria. By the latter, he understands glucose that has not passed the liver, and has consequently not been formed into glycogen. It is the glucose that is taken up by intravenous ad-

¹ Markuse Über die Bedeutung der Leber für das Zustandekommen des Pankreasdiabetes, *Zeitschr f klin Med*, LVII, p 225, 1894

Montuori Sull'importanza del fegato nel diabete pancreatico, *Gazz d Osped ed clin*, 1895, no 16

³ Rosenfeld Die Oxydationswege des Zuckers, *Berl klin Wochenschr*, LV, 1907

ministration or the glucose that circulates after the extirpation of the liver or Eck's fistula. These conclusions apparently fit in with other experiments of the author in which he showed that more glycogen arises in the liver after feeding of glucose *per os*, than after injecting intravenously. He also found that phlorhizinized animals eliminate more glucose after feeding of glucose *per os* than after subcutaneous injection. These experiments are of such great importance in the physiology of glucosuria, that a repetition of them seemed desirable.

Five dogs were operated upon. The method of operation was described by one of us (Sweet)⁴. All dogs were brought to autopsy and the existence of the fistula verified. The animals were phlorhizinized in the usual manner, and in none of the animals did the phlorhizin fail to bring about glucosuria, nor was there any deviation from the usual course. The outcome of our experiments undermines the very foundation of Rosenfeld's theory, and an analysis of his other experiments gives them an entirely different meaning. He gave one phlorhizinized dog 100 grams of glucose *per os*. Seventy-eight grams of glucose were recovered in the urine. The same dog, after it had overcome the effects of phlorhizin, received another 100 grams of glucose intravenously. This caused a temporary glucosuria with the elimination of 21 and 23 grams of glucose in two cases. The animal was then phlorhizinized as before and 100 grams of glucose given intravenously. Only 36 grams of glucose appeared in the urine, 15 grams more than normally. This led Rosenfeld to the conclusion "*dass die intravenos gegebene Glucose von diabetischen Hunden unvergleichlich besser vertragen wird als dieselbe Glucose wenn sie per os aufgenommen wird*". He reasons that the orally-fed glucose gives rise to glycogen and hence the glucose that comes from it is not burnt readily and is thrown out in larger quantities in glucosuria, whereas the intravenously-given glucose does not yield glycogen, and hence it is burnt.

In the light of our present understanding of phlorhizin glucosuria, it does not seem necessary to explain so simple an experiment in so speculative a way. It is very well established now that in phlorhizin glucosuria the animal does not lose the power

⁴ Sweet. The Artificial Anastomosis between the Portal Vein and the Vena Cava Inferior—Eck's Fistula, *Journ of Exp Med*, vii, p 2, 1905

of burning glueose The amount that may be burnt is a function of the ratio between the velocity of absorption of glueose and the velocity of its excretion by the kidneys In other words, the amount burnt depends upon the amount that is circulating in the blood Assuming the ratio of excretion to be constant, as was probably the case in Rosenfeld's dog, conditions for the combustion of sugar were certainly more favorable in the case of the intravenous administration, for there we have a sudden increase in the glucose concentration of the blood, which cannot possibly be removed by the kidneys at the rate at which it is supplied Whereas in the administration of glueose *per os*, the absorption is a very slow one, small quantities of glucose enter the blood at a time, and it is swept out by the kidneys more completely It is a very common experience that small quantities of glucose are recovered much more quantitatively than are large quantities

From the aforesaid, there seems to be no reason for the acceptance of Rosenfeld's hypothesis

Finally, we tested the extent to which the throwing out of the liver by Eck's fistula influences gluconeogenesis One of our phlorhizinized dogs received 15 grams of glycocoll at one time and 5 grams at another time Here again, no deviation from the normal could be obtained The amount of "extra glueose" is similar to that obtained under ordinary conditions (Ringer and Lusk)⁵

Dog with Eck's fistula Phlorhizin glucosuria

Twelve hour periods

DATE 1912 APRIL	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D N	NH ₃ N	ACETONE AND ACETO ACETIC ACID	β HYDROXY BUTYRIC ACID	REMARKS
24	I	15 08	6 48	23 32	3 60	0 37			
24	II		6 96	25 10	3 61	0 45	0 14	0 89	{ 15 gm glycocoll subcutan
25	III	14 56	8 69	34 87	4 01	0 32	0 12	0 84	
25	IV		5 75	20 52	3 57	0 42	0 22	1 44	
26	V	14 20	5 24	16 85	3 21	0 40	0 25	1 56	{ 5 gm glycocoll as above
26	VI		6 61	22 89	3 46	0 44	0 21	1 33	
27	VII	14 00	5 48	16 70	3 05		0 20	1 18	

⁵ Ringer and Lusk Über die Entstehung von Dextrose aus Aminosäuren bei Phlorhizinglykosurie, *Zeitschr f physiol Chem*, lxvi, p 106, 1910

SUMMARY.

Eck's fistulae were performed on five dogs. The dogs were kept from one week to two months, when phlorhizin was administered. The glucosuria that followed resembled in every detail that observed in normal dogs. This is contrary to the findings of Rosenfeld, who did not obtain any glucosuria at all.

The power of gluconeogenesis is not diminished in dogs with Eck's fistula.

THE INFLUENCE OF PHLORHIZIN ON A SPLENECTOMIZED DOG

By J H AUSTIN AND A I RINGER

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(Received for publication, January 30, 1913)

In a paper on the action of tissues and tissue juices on glucose, Levene and Meyer¹ found that the spleen possesses an activator for the enzyme which causes a condensation of the glucose molecule in muscle, liver, pancreas and lung. One of us (Austin) had several splenectomized dogs at his disposal, and we thought it of interest to see whether the absence of the spleen would in any way modify the course of the glucosuria after phlorhizin administration. The results were entirely negative, *i e*, the glucose elimination, the D/N ratio and the degree of acidosis resembled in every respect the course of glucosuria in normal dogs.

¹ This *Journal*, xi, p 356, 1912

A METHOD FOR DETERMINING THE SURFACE TENSION OF LIQUIDS FOR BIOLOGICAL PURPOSES

By C C ERDMANN

(From the Chemical Laboratory of McLean Hospital, Waverley, Mass)

(Received for publication, January 31, 1913)

In several papers, I Traube¹ gives an account of interesting results obtained by means of capillary analysis. He emphasizes the importance of this method for biological work, since it enables us to observe reactions between components present in quantities so minute that gravimetric or volumetric methods utterly fail to demonstrate them. His apparatus, the stalagmometer, is simple in construction. It consists of a pipette whose outlet is formed by a short capillary terminating in an enlarged disc so as to offer a plane, polished surface for the formation of the drop. The resistance encountered by the liquid in passing the capillary adjusts the delivery of the drops so that, according to the viscosity of the liquid, about three to four seconds are required for their formation. Two marks on the pipette define a certain volume, and the number of drops contained in this volume is counted by an automatic device. Having established the drop number of the apparatus for distilled water, the surface tension relative to water of a liquid can easily be determined.

Trying to carry out the same idea in a different way, the following modification was adopted. A drop pipette for the delivery of a certain number of drops whose weight is to be considered is the essential part of the apparatus. The drop pipette, of a few cubic centimeters capacity, so as to enable determinations when small amounts only of substance are obtainable, is provided with a highly polished drop surface ground so as to form the base of a cone. The flow of the liquid is regulated by a narrow capillary (0.2-0.3 mm in diameter) and thus, for the formation of the

¹ *Ber d deutsch chem Gesellsch*, p 44, 1911, *Biochem Zeitschr*, *xxv* p 341, 1910

drops, about five seconds are required. Drops delivered from this capillary were found to be uniform, so far as could be judged by their weight, provided that the drop surface was clean and the drop contained no air bubbles.

The details of the apparatus (made by Eimer & Amend of New York) may be seen in figure 1 of the accompanying sketch. *A*, polished drop-surface of about 5 mm in diameter, *B*, a thin capillary of about $\frac{1}{2}$ mm, *C*, a wider capillary of about 1 mm, *D*, bulb of several cubic centimeters capacity (containing about 60 drops), *E*,

short capillary with mark *F*, then ordinary glass tubing narrowed at *G* in order to hold in place the absorbent cotton *H* which excludes dust or particles of the stopper *I* from getting into the pipette, *K*, connection with the outside air, *L*, rubber stopper to connect the weighing bottle with the apparatus. The pipette is embedded in a water jacket for the maintenance of a uniform temperature.

The water jacket consists of an inverted Winchester bottle cut so that the neck part contains about 1.5 liters. The pipette is inserted in the neck by means of plaster of paris, and a layer of mercury protects the latter from the action of the water.

A thermometer indicates the temperature of the water jacket, and by addition of either hot or cold water a certain temperature can be maintained for some time.

Though slight differences in temperature do not materially change the size of the drops, for accurate determinations the observation of the different temperatures is important, and therefore a thermostat arrangement adjusted so as to correspond to the average room temperature may be used.

The determination of the surface tension of a liquid is carried out as follows: the clean pipette is first rinsed with the liquid by passing a small amount into the bulb and carefully drawing air through it. The short capillary will prevent the liquid from coming into contact with the absorbent cotton. Then the pipette

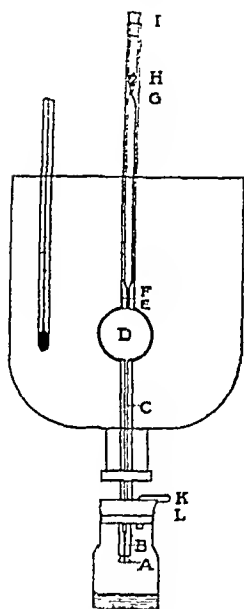


FIGURE 1

is filled, closed with a stopper, and about fifteen minutes are allowed for the exchange of temperature differences between the water jacket and the contents of the pipette. In the meantime the drop-surface is cleaned with fat-free filter paper. Several drops are allowed to pass, the presence of air bubbles is easily detected and eliminated and, when it is safe to assume that the protruding part of the pipette has accepted the water jacket's temperature, the pipette is closed again, and the weighing bottle attached to the apparatus by means of the rubber stopper. The weighing bottle, which has a capacity of about 50 cc, contains a layer of liquid paraffin to prevent evaporation. The withdrawal of the stopper of the pipette will start the formation of the drops, and thus 10, 20 or more drops are collected. It was found unnecessary to count the number of drops for a liquid more than once, since a certain weight corresponds to a certain number of drops.

In collecting 10 drops of the same liquid in each of two weighing bottles, the maximum difference in their weight will be found not to exceed 0.0005, the weight of a drop of water, for instance, being 0.0850 gram, this difference would amount to $\frac{1}{170}$ of a drop. Using a thermostat arrangement, in about twenty-five determinations it was found that for distilled water the maximum difference (≈ 0.0012) was due to the varying purity of the distilled water obtained from the laboratory still as well as to differences between the thermostat and the room temperatures.

As there is a possibility of determining the viscosity of the liquid at the same time, the use of a stop-watch was found convenient, especially for those determinations in which a larger number of drops was collected. In order to facilitate the comparison of the results obtained from different liquids, these results may be expressed as *drop numbers*, corresponding to 5, 10 or 50 grams of substance.

The following experiments were carried out by means of a drop pipette which was surrounded by a water jacket without thermostat.

A solution of serum (rabbit) was prepared, 1:100 in 0.85 per cent sodium chloride. Having established its drop number, to 50 cc of serum were added 1, 2, 5 and 10 cc of $\frac{N}{100}$ acid in the one line of experiments and the same amount of alkali in the other. After standing for about twelve hours at room temperature, the solutions were filtered (Schleicher & Schüll, 591) and 1, 2 or 3 times 10 drops were collected. The weight of 10 drops divided into 50 (grams of substance) furnished the following drop numbers

Temperature of the water jacket = 16.2° - 16.4°

SUBSTANCE	WEIGHTS (GRAMS) FOR 10 DROPS	DROP NO
0.85 per cent NaCl solution	0.8263, 0.8266	605.0
50 cc + 5 cc $\frac{N}{100}$ NaOH	0.8253 (not filtered)	605.7
50 cc + 5 cc $\frac{N}{100}$ HCl	0.8262 (not filtered)	605.1
Serum 1 100	0.8213, 0.8212, 0.8213	608.9
From another rabbit	0.8214, 0.8213	608.9
The same 3 days later	0.8212	609.0
The same 7 days later	0.8051 (decomposed)	621.0
Serum + $\frac{N}{100}$ HCl		
50 cc serum + 1 cc	0.8185, 0.8187, 0.8186	610.9
50 cc serum + 2 cc	0.8038, 0.8038, 0.8038	622.1
50 cc serum + 5 cc	0.7820, 0.7820, 0.7821	639.5
50 cc serum + 10 cc	0.7511, 0.7518, 0.7517	665.5
Serum + $\frac{N}{100}$ NaOH		
50 cc serum + 1 cc	0.8226, 0.8226, 0.8226	607.8
50 cc serum + 2 cc	0.8212, 0.8213, 0.8212	609.0
50 cc serum + 5 cc	0.8173, 0.8176, 0.8173	611.7
50 cc serum + 10 cc	0.8130, 0.8127, 0.8128	615.0

The change in the surface tension of the NaCl solution produced by the addition of either NaOH or HCl is so slight that it can be ignored. While small amounts of acid cause a pronounced change in the surface tension of serum, it shows a certain tolerance for alkali.

Results obtained from cerebro-spinal fluid treated in the same way

SUBSTANCE	DROP NO
Water	584.3
Undiluted fluid	615.7
1% solution cerebro-spinal fluid in 0.85% NaCl	606.7
Later determinations	607.3
Fluid decomposing	608.1
50 cc of fluid 1 100 + $\frac{N}{100}$ HCl	
1 cc	607.5
5 cc	608.3
50 cc of fluid 1 100 + $\frac{N}{100}$ NaOH	
1 cc	607.7
5 cc	607.8

The next determinations were carried out on syphilitic and non-syphilitic sera (which I owe to the kindness of Dr. James

H Wright of the Massachusetts General Hospital) subjected to the Noguchi modification of the Wassermann reaction

0.85 per cent NaCl solution = 639.4

NO	DROP NUMBERS FOR 50 GRAMS OF SUBSTANCE		REACTION
	Incubated	Not incubated	
2	646.0	645.1	Negative
7	645.5	654.6	Strongly positive
10	642.2	643.6	Positive
13	646.6	644.7	Slightly positive
14	644.8	644.8	Negative
16	655.5	655.6	Negative
17	644.6	644.5	Negative
18	658.1	657.6	Strongly positive

For the surface tension determination, the sera were diluted 1:125 with salt solution, except numbers 16 and 18, which were used in a more concentrated solution (about 1:50). The differences in surface tension before and after incubation, expressed as drop numbers for 50 grams of substance, are very slight, hardly exceeding the experimental error, and in accordance with the investigations of Bertolini² who was unable to obtain results analogous to those of Ascoli.³

For surface tension determinations of less dilute solutions, a modification of the above pipette was used (see figure 2), which, not being surrounded by a water jacket, offered the possibility of introducing the liquid directly into the bulb by means of a short side tube. This side tube is closed by a rubber stopper holding a thermometer. Instead of filtering the solutions they are centrifuged for about fifteen minutes at high speed, thus small solid particles are removed which would be liable to be retained in the small capillary. Before the collection of the drops is begun, fifteen minutes are allowed for the acceptance of room temperature, a slight



FIGURE 2

² *Biochem Zeitschr*, xxviii, p. 60

³ *Münch med Wochenschr*, 11, p. 62, 1910

pressure on the rubber bulb will start the formation of the drops. The temperature coefficient having been established, all results can be calculated for an average temperature.

The effect of heating on cow serum

About 500 cc of cow serum contained in a long-necked bottle were shaken in a water bath, the temperature of which was slowly raised. A thermometer immersed in the serum indicated its temperature, and from time to time (each additional 5°) a small amount was withdrawn, until coagulation rendered this impossible, and set aside for the surface tension determination. The following table indicates the results expressed in drop numbers for 50 grams of substance.

For another experiment cow serum was used to which a small amount of sodium benzoate had been added and which had been standing for three days in an ice chest.

TABLE I
Average room temperature 20°

TEMPERATURE	WEIGHT OF 10 DROPS	DROP NO	TEMPERATURE	WEIGHT OF 10 DROPS	DROP NO
deg C			deg C		
Room	0.7688	650.7	45	0.7618	656.5
25	0.7674	651.6	50	0.7589	659.0
30	0.7636	655.0	55	0.7538	663.2
35	0.7628	655.4	60	0.7456	670.4
38	0.7629	655.4	65	0.7136	700.3
40	0.7632	655.3	70	0.6716	744.5

In both experiments the change in surface tension was found to correspond to a curve rising slowly at lower temperatures but rapidly at the beginning of coagulation, from 65°-70°. At 38°, however, a deviation occurs apparently caused by enzymes, which, at temperatures nearing that of the body, find the conditions of optimum efficiency. It was noticed that the serum, though kept in an ice chest, showed daily changes in surface tension, and therefore to a new preparation of serum about 0.1 per cent of sodium benzoate was added to prevent bacterial decomposition. Nevertheless, a similar change in surface tension occurred, and enzyme action must be held responsible for it. This would also explain

TABLE II
Average room temperature 20°

TEMPERATURE	WEIGHT (10 DROPS)	TIME FOR FORMATION OF 9 DROPS	DROP NO (50 GRAMS)
<i>deg C</i>		<i>seconds</i>	
Room	0 7643	119	654 1
30	0 7640	115	654 5
35	0 7629	115	655 5
38	0 7633	115	655 1
40	0 7617	115	656 4
45	0 7610	115	657 0
55	0 7603	114	657 6
60	0 7547	116	662 4
65	0 7368	124	678 6
70	0 6928	219 for 4 drops	722 0

the fact that the surface tension is influenced by freezing and subsequent thawing of the serum. Changes in the viscosity of the serum are noticeable only when it has been heated nearly to coagulation temperature.

ON THE ACTION OF LEUCOCYTES ON SOME HEXOSES AND PENTOSES

THIRD COMMUNICATION

CONTRIBUTION TO THE MECHANISM OF LACTIC ACID FORMATION FROM CARBOHYDRATES

By P A LEVENE AND G M MEYER

(From the Rockefeller Institute for Medical Research, New York)

(Received for publication, February 3, 1913)

In previous communications the authors have demonstrated that, through the action of leucocytes, glucose is transformed into d-lactic acid, and that the cleavage of the sugar molecule does not proceed beyond this phase. Since then, the results obtained by us were corroborated by Embden and his co-workers, Kondo and K v Noorden, Jr,¹ and by Rona and Arnheim.²

Regarding the conditions of the experiments it was stated that a certain degree of hydroxyl concentration, such as is offered by a 1 per cent Henderson phosphate mixture, was required for a successful result, and that distilled water could not be used as a medium of the reaction, since it prevented the reaction from taking place, and antiseptics, such as toluene and chloroform, acted in the same way. At the time of our first publication we had overlooked a statement of Rona and Doblin³ that the glycolysis generally produced by blood was absent when blood had been diluted with distilled water or when chloroform had been added to it.

The observations on the action of leucocytes were extended to a larger number of sugars, both hexoses and pentoses, with a view of elucidating the mechanism by which, in the organism, lactic acid is formed from sugar. It is obvious that a molecule of hexose cannot undergo a direct decomposition into two mole-

¹ *Biochem Zeitschr*, xlv, pp 63 and 94, 1912

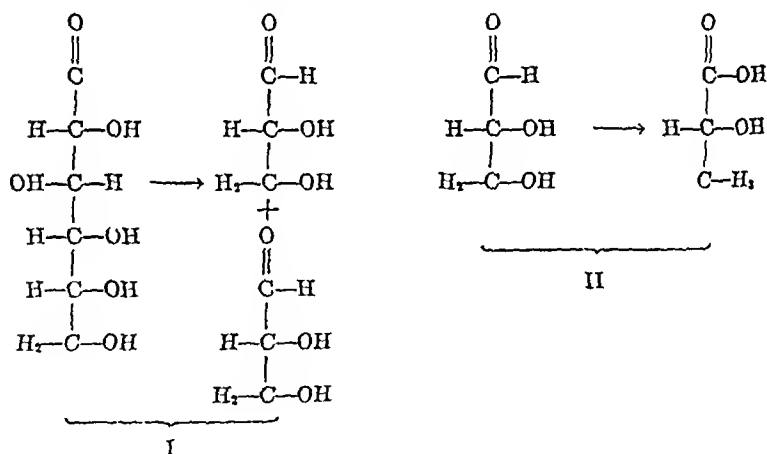
² *Ibid*, xlviii, p 35, 1913

³ *Ibid*, xxxii, pp 489-508, 1911

150 Lactic Acid Formation from Carbohydrates

cules of lactic acid without passing a number of intermediary transformations In recent years there have been brought to the front a great many speculations tending to elucidate the mechanism of the reaction They can be found in recent publications on alcoholic fermentation, lactic acid fermentation or on glycolysis

After the publication of our previous articles on the action of leucocytes on glucose, and while the present investigation was in progress, there appeared a series of articles by Embden and his co-workers, on the basis of which Embden formulated a theory that by the action of tissue enzymes one molecule of glucose is dissociated into two molecules of glyceric aldehyde, second, that glyceric aldehyde is then transformed into lactic acid, and third, that the α -carbon atom of the glyceric aldehyde remains unaffected in course of the transformation The mechanism may be presented in the following form

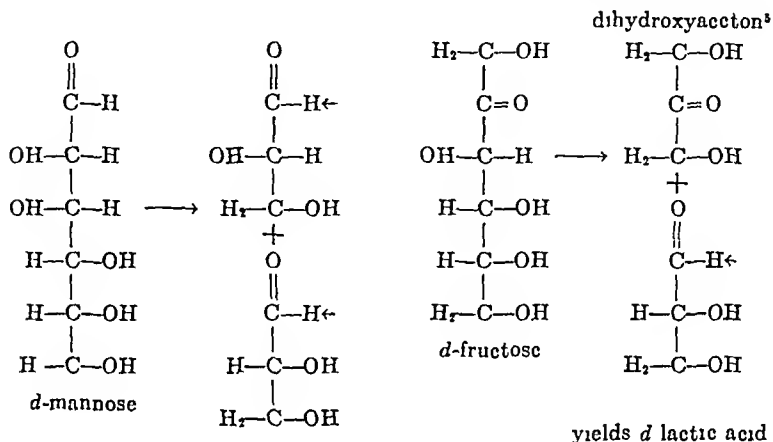


Although our own work on the mechanism of the reaction is not yet completed, yet it seems to us, that it furnishes a very definite argument against the correctness of Embden's view⁴

It is obvious that if a molecule of glucose dissociates into two of lactic acid, according to the hypothesis of Embden, both of the latter possess the same optical character Thus, *d*-glucose may yield two molecules of *d*-lactic acid In this case, however,

⁴ Biochem Zeitschr., *iv*, p 108, 1912

mannose and fructose would not be expected to yield the same result. It is evident from a glance at the configuration of the respective sugars that mannose would be expected to form *dl*-lactic acid, and fructose a mixture of *dl*- and *d*-lactic acid.



yields *dl*-lactic acid

It was found in the course of our experiments that fructose, mannose and galactose all are transformed into lactic acid by means of leucocytes under the conditions reported in the previous communications. Regardless of the nature of the hexose, the lactic acid formed was invariably the *d*-form. Of course, it is important to make certain that the lactic acid obtained in the experiment did not consist of a mixture of the active and inactive forms. This possibility can be excluded in our experiments on the following grounds: first, the zinc salt of the *dl*-lactic acid is more insoluble than that of the optically active form, hence, it should be the first to crystallize if it were present in the mixture, second, the specific rotation of the zinc salt obtained from any one of the hexoses was of the same magnitude. Thus, on the basis of these experiments it does not seem possible that the formation of lactic acid is brought about by simple rearrangement in the molecule of glyceric aldehyde. Whether or not pyruvic aldehyde is the phase immediately preceding the formation of lactic acid remains to be established.

² Would be expected to yield *dl*-lactic acid

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Attempts to bring about a dissociation of pentoses by means of leucocytes resulted negatively

It may be mentioned here that tissues preserved under strict aseptic conditions act on sugars in a way identical to leucocytes. The results of the experiments in that connection will be communicated separately

EXPERIMENTAL

Leucocytes These were obtained from dogs by injections of turpentine into the pleural cavity. The technique and further handling of the material has been described in a previous communication

Solutions The suspensions were made in the identical manner as the glucose experiments, in a 1 per cent Henderson phosphate solution

Bacteriological controls Both aerobic and anaerobic cultures were made from all leucocyte-sugar mixtures at the close of the experiment and only those analyzed which were sterile. The bacteriological examinations were made by Dr J Bronfenbrenner and we take this occasion to express our appreciation

Methods of analysis Sugar was estimated by reduction of Fehling's solution, the reduced copper was determined by Volhard's method

Lactic acid Since the previously reported experiments we have succeeded in obtaining a von der Heide ether extraction apparatus⁶. The leucocyte sugar solutions were carefully made neutral to litmus, brought to a boil and the proteins coagulated with the addition of very dilute phosphoric acid. The addition of sodium sulphate crystals facilitates the precipitation of the proteins. The solution was filtered and again made neutral to litmus, and then sufficiently concentrated *in vacuo* to be contained in the ether extractor. Crystalline sodium sulphate is added to give about a one-half saturated solution and then 5 to 10 cc of phosphoric acid are added. Extraction is allowed to proceed for at least seventy-two hours. The ether extract is then dried over anhydrous sodium sulphate and filtered. Water is added to the flask and the ether distilled off. The lactic acid in aqueous solution is converted into the zinc salt in the usual manner

⁶ Bericht d. k. ö. m. g. l. Lehranstalt f. Weinbau in Geisenheim a. Rh., 1906,

I Experiments showing disappearance of sugars in mixture of sugar and leucocytes

Mannose

	SOLUTION USED	NH ₄ CNS	NH ₄ CNS PER CC	SUGAR	LOSS	PER CENT LOSS
	cc	cc		percent		
a At beginning of experiment	1	20.6	20.6	6.35		
After thirty-six hours	1	19.8	19.8	6.10	0.25	4.10
b At beginning of experiment	2	36.4	18.2	5.62		
After thirty-six hours	2	34.8	17.4	5.36	0.26	4.86
c At beginning of experiment	1	13.6	13.6	4.19		
After thirty-six hours	1	12.0	12.0	3.69	0.50	11.90
d At beginning of experiment	1	20.0	20.0	6.16		
After thirty-six hours	1	17.6	17.6	5.42	0.74	12.00
e At beginning of experiment	1	19.0	19.0	5.85		
After thirty-six hours	1	16.3	16.3	5.03	0.82	14.00

Lactulose

a At beginning of experiment	2	37.2	18.6	6.40		
After thirty-six hours	2	35.4	17.7	6.09	0.31	5.10
b At beginning of experiment	2	35.0	17.5	6.02		
After thirty-six hours	2	33.4	16.2	5.56	0.46	7.35
c At beginning of experiment	1	17.6	17.6	6.05		
After thirty-six hours	1	16.4	16.4	5.48	0.53	8.40
d At beginning of experiment	1	15.9	15.9	5.41		
After thirty-six hours	1	14.5	14.5	4.90	0.51	8.95

Galactose

a At beginning of experiment	2	30.8	15.4	5.61		
After thirty-six hours	2	29.8	14.9	5.43	0.18	3.20
b At beginning of experiment	2	28.0	14.0	5.09		
After thirty-six hours	2	26.2	13.1	4.77	0.32	6.29

Arabinose

a At beginning of experiment	2	29.2	14.6	5.69		
After thirty-six hours	2	29.2	14.6	5.69	0	0
b At beginning of experiment	2	28.2	14.1	5.50		
After thirty-six hours	2	28.4	13.2	5.53	0	0

Xylose

a At beginning of experiment	2	27.6	13.8	5.38		
After thirty-six hours	2	27.6	13.8	5.38	0	0

154 Lactic Acid Formation from Carbohydrates

II Experiments showing formation of d-lactic acid during "Glycolysis" of mannose

a 150 cc of the leucocyte mannose mixture (c, Experiment I) were extracted in a von der Heide extractor with ether Yield of crude lactic acid = 0.2134 gram

0.145 gram of the recrystallized salt in 2.167 grams of water in a 1 dm tube gave a rotation, $\alpha = -0.45^\circ$

$$[\alpha]_D^{20} = -6.7^\circ$$

b 300 cc of leucocyte mannose mixture (d and e, Experiment I) were together extracted with ether in a von der Heide extractor

0.563 gram anhydrous zinc lactate was obtained This was recrystallized and analyzed

0.2278 gram of the recrystallized salt lost, on drying to constant weight at 110° ,

0.029 gram H_2O = 12.73 per cent H_2O

Calculated for two molecules H_2O = 12.88 per cent

0.0906 gram anhydrous salt after ignition

gave 0.0302 gram ZnO = 33.33 per cent ZnO

Calculated = 33.40 per cent

0.1344 gram zinc salt in 1.8684 grams H_2O gave a rotation in a 1 dm tube of $\alpha = -0.47^\circ$

$$[\alpha]_D^{20} = -7.0^\circ$$

c 300 cc of laevulose leucocyte mixture (c and d) were extracted with ether in a von der Heide extractor The yield of recrystallized zinc lactate = 0.2474 gram

0.1624 gram recrystallized salt lost, on drying

to constant weight, 0.0211 gram

H_2O = 12.95 per cent H_2O

Calculated for two molecules H_2O = 12.88 per cent

0.1413 gram anhydrous salt was ignited and

gave 0.0469 gram ZnO = 33.20 per cent ZnO

Calculated = 33.40 per cent

0.1536 gram zinc salt in 2.0138 grams H_2O gave a rotation in a 1 dm tube of $\alpha = -0.49^\circ$

$$[\alpha]_D^{20} = -6.8^\circ$$

AN ENZYME CONCERNED WITH THE FORMATION OF HYDROXY ACIDS FROM KETONIC ALDEHYDES

BY H D DAKIN AND H W DUDLEY

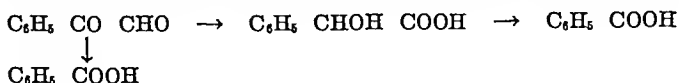
(*From the Herter Laboratory, New York*)

(Received for publication, February 3, 1913)

Since so little is known of the unstable labile substances which actively participate in the synthetic reaction of the living cell, and as it is so difficult to frame adequate experiments for the analysis of these various reactions, it seemed desirable to follow some of the changes undergone by structurally related substances which have been rendered partially stable by the incorporation of an aromatic group

The present paper deals with the fate of phenyl glyoxal in the animal body and its decomposition by animal tissues

Phenyl glyoxal administered to rabbits in doses of 1-1.5 grams per kilo leads to the excretion of about half a gram of optically active *l*-mandelic acid and about three-quarters of a gram of hippuric acid. No phenyl glyoxylic acid was detected. The fact that a ketonic aldehyde through enzyme action may unite with water to form an optically active hydroxy acid appears to be of some significance. The benzoic acid (hippuric acid) may originate either by the direct oxidation of the phenyl glyoxal or by the oxidation of mandelic acid or probably by both reactions



An examination of the action of aqueous extracts of various animal tissues, including liver, pancreas, heart, skeletal muscle, kidney, blood, spleen and brain, showed that in every case an enzyme was present capable of converting phenyl glyoxal into mandelic acid and that the action of the catalyst was readily inhibited by heat. It would seem, therefore, as if the reaction was a rather general one

The rôle that ketonic aldehydes may play in metabolism is not yet clear, but it is at least suggestive to recall that methyl glyoxal is readily obtained by the decomposition of sugar by mild hydrolysis and that by analogy methyl glyoxal should yield lactic acid, when acted upon by the enzyme previously referred to. Conversely, should the enzyme reaction prove to be reversible, as seems likely, may not this type of reaction be concerned with the actual formation of carbohydrate from lactic acid and indirectly from amino-acids? The possibility of amino-acid synthesis from methyl glyoxal must also be considered



The testing of the above hypothesis involves somewhat difficult experiments and will take considerable time

It is interesting to note that phenyl glyoxal readily combines with histidine, arginine, ornithine and lysine to give sparingly soluble yellow substances. These compounds are under investigation

EXPERIMENTAL

Three experiments were made in which phenyl glyoxal (15 grams) dissolved in warm water (80 cc) was given by stomach tube to rabbits weighing about 15 kilos. The urine in each case was collected for about twenty-four hours and proved to be distinctly laevorotatory owing to the presence of mandelic acid. On distilling the urine, no evidence was obtained of the presence of unchanged phenyl glyoxal. The urines were acidified with phosphoric acid and extracted with ether in a continuous extractor. On concentrating the ether extract, from 1.3–1.5 grams of crystalline residue were obtained. This was dried on porous plates and the bulk of the hippuric acid obtained by direct crystallization from water. The hippuric acid melted at 185° and gave 7.7 per cent of nitrogen on analysis (calculated 7.8 per cent).

The mother liquor from the hippuric acid was shaken thrice with small portions of ether. The ether extract on evaporation gave crude mandelic acid (0.4–0.5 gram) which was readily purified by recrystallization from benzene and was completely free from hippuric acid.

In each case, some inactive mandelic acid (20 per cent) was found with the active variety, the former being probably formed by the direct hydrolysis of phenyl glyoxal other than by enzyme action. On repeated recrystallization from water the more sparingly soluble active acid, m p 130–132°, separates first

0.1221 gram gave 0.2830 gram CO_2 and 0.0595 gram H_2O

ANALYSIS

	Found	Calculated for $\text{C}_8\text{H}_8\text{O}_3$
C	63.2	63.2
H	5.4	5.3

For the investigation of the action of tissues upon phenyl glyoxal, mixtures were made of from 20 to 50 grams of minced tissue with a solution containing 0.1 to 0.25 gram of phenyl glyoxal, with half its weight of sodium bicarbonate. After incubation at 37° for about twenty-four hours in the presence of toluene, the solutions were heated on a water bath with an equal volume of saturated ammonium sulphate solution containing a little phosphoric acid. The mandelic acid was extracted by shaking with ether, and the ether extract taken up in cold water (10 cc), filtered and examined in the polarimeter in a 2 dm tube. In every case laevo rotations of from 0.8° to 2.1°, due to *l*-mandelic acid, were observed. The mandelic acid was readily obtained in crystalline form for identification. Experiments in which the tissue extract was boiled prior to adding the phenyl glyoxal showed rotations of less than 0.18°. The results indicated that from 40 to 60 per cent of the phenyl glyoxal was converted into laevomandelic acid in every case.

A more detailed study of the action of the enzyme is in progress.

NOTE ADDED AT PROOF CORRECTION Further investigation has shown that the enzyme above referred to is active in aqueous extracts of various tissues and that it may be roughly purified by precipitation with salts. An enzyme solution prepared from dog's liver when added to pure methyl glyoxal (4 grams), prepared according to Meisenheimer's method, effected its complete decomposition in less than ten minutes with formation of lactic acid.

TANNIC ACID FERMENTATION I

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I PREFACE

During 1907-1908, a preliminary investigation of the conditions of tannic fermentation was made by the writer with the purpose of improving the practical methods involved. In the course of this preliminary investigation a number of interesting observations were made, justifying a further study of the conditions of the fermentation and of the relation of various organisms to the process. With the progress of the investigation other phases suggested themselves, until ultimately four distinct but correlated parts of the subject were experimentally studied. Part I, here reported, includes chiefly (1) the toxicity of tannic acid for various fungi, (2) a comparison of the organism *Aspergillus niger* and *Penicillium sp* in the fermentation of tannic acid, (3) the conditions and influence of various factors on the fermentation process. Part II is concerned primarily with the influence of nutrition on the production of the enzyme tannase, and will be reported in a subsequent paper.

These investigations were begun at the suggestion of Prof B M Duggar and prosecuted in his laboratory. It is a pleasure here to express my thanks for the advice, kindly criticism and assistance, which he has so generously given.

II INTRODUCTION

Chemical nature of tannic acid Wagner¹ has grouped the tannins into a "physiological" and a "pathological" series, the latter including, as most important, the tannin of oak galls as

¹ R Wagner Beiträge zur Kenntnis und zur quantitativen Bestimmung der Gerbsäuren, *Zeitschr f anal Chem* v, pp 1-10, 1866

well as the tannin of sumach and chestnut. The pathological tannins are hydrolyzed by boiling with acids or through the action of the enzyme tannase, gallic acid resulting. For other distinguishing characteristics of the tannins Trimble's² and Proctor's³ treatises may be consulted, and for a discussion of the diverse views held regarding their chemistry reference should be made to special papers on the subject.

This paper is concerned with the fermentation of the tannin from oak galls, which is frequently termed gallotannic acid.

History of tannic acid fermentation. Schceele⁴ found in 1786 that gallic acid was present in the gall nuts. Robiquet⁵ attributed the fermentation of the gall nuts to a ferment within the gall nut. Laroque⁶ considered the formation of gallic acid from tannic acid to be due either to a ferment or to oxidation. He further found that various toxic substances could inhibit the fermentation. Ed Robiquet⁷ showed that the tannic acid was transformed during the fermentation and he believed the transformation to be due to the ferment pectase which he extracted from the gall nuts. Wittstein⁸ stated that beer yeast aided tannin fermentation by fermenting the sugars and other products present. Van Tieghem⁹ was the first, however, to demonstrate that the formation of gallic acid during fermentation is due to the action of fungus organisms, and not to enzymes pre-existing in the galls, nor to oxidation by the air. He stated further that the organisms were *Penicillium glaucum* and a new organism which he named *Aspergillus niger*. He found that if the growth was submersed, the tannic acid was converted into gallic acid and glucose, the glucose being gradually used up, the gallic acid remaining. He stated further that if the growth was on the surface, sporulation and greater growth occurred and that the tannic acid was destroyed directly, the slight hydrolysis being due to submerged mycelium, the resulting glucose and gallic acid being then assimilated.

² H. Trimble, *The Tannins*, Part I, 168 pp., Part II, 172 pp., 1892.

³ H. K. Proctor, *Leather Industry Laboratory Book*, 2nd edition, 450 pp., 1908.

⁴ Quoted from H. Trimble *loc cit*.

⁵ M. Robiquet, *États pour servir à de l'acide gallique*, *Ann. de chim. et de phys.*, 2^e série, lvi, pp. 385-409, 1837.

⁶ A. Laroque, *Neue Untersuchungen über Gallussäure*, *Ann. d. Chem. u. Pharm.*, xxxix, pp. 37-100, 1841.

⁷ Ed. Robiquet, *Recherches sur la fermentation gallique*, *Ann. de chim. et de phys.*, 3^e série, xxxix, pp. 453-460, 1853.

⁸ Wittstein, *Jahresber. über die Fortschritte der Chemie*, 1853, p. 435.

⁹ Ph. Van Tieghem, *Sur la fermentation gallique*, *Compt. rend. de l'Acad. des Sci.*, lxxv, pp. 1091-95, 1867.

Muntz¹⁰ found that fermentation occurred through the action of *Penicillium glaucum*. Fernbach¹¹ grew *Aspergillus niger* in Raulin's solution with the sugar replaced by tannic acid, and then extracted from the organism the enzyme tannase. Pottevin,¹ in a similar manner and at the same time, extracted the enzyme tannase from the same fungus. He noted also that the enzyme was developed when *Aspergillus niger* was grown on Raulin's solution with the sugar replaced by gallic acid. He stated that the tannase acted on tannate of gelatin and also hydrolyzed methyl salicylate and ethyl salicylate.

Manca¹³ showed that synthetically prepared digallic acid was not split up by *Aspergillus niger* and *Penicillium glaucum* into gallic acid, and therefore concluded that the hydrolyzable tannin of the gall nut could not be a digallic acid. The latter in high concentrations was toxic to the organism. Further, Manca estimated quantitatively the digallic and tannic acid used by each organism. In a study of the fermentation process he employed pure cultures, adding a previously sterilized Raulin's solution rendered strongly acid. The quicker the fermentation, the richer was the yield of gallic acid obtained.

Kunz-Krause¹⁴ found an octyl gallotannoid, $C_{84}H_{80}O_{33}$, which through the action of a mould was transformed to gallic acid.

III. METHODS

Culture solution Throughout all the work the culture solutions used were a slight modification of Richards'¹⁵ solution or of Czapek's solution.¹⁶ These solutions are designated respectively A and B and are as follows:

¹⁰ Muntz *Ber d deutsch chem Gesellsch*, 1877, p 1773

¹¹ A. Fernbach *Sur la tannase*, *Compt rend de l'Acad des Sci*, cxxxi, pp 1214-15, 1901

¹² H. Pottevin *La tannase. Diatase dedoublant l'acide gallotannique*, *Compt rend de l'Acad des Sci*, cxxxi, pp 1215-17, 1901

¹³ A. Manca *Sur les acides gallotanniques et digaliques*. These, Geneva, 1904. (Cited from Lafar *Handb d technische Mykologie*, I, p 663.)

¹⁴ Kunz-Krause *Fragmente zu einer Monographie d Tannoide*, *Pharm Centralbl*, Halle, 1898. (Cited from Lafar *Handb d technische Mykologie*, I, p 662.)

¹⁵ H. M. Richards *Die Beeinflussung des Wachstums einiger Pilze durch chemische Reize*, *Jahrb f wiss Bot*, xxv, pp 665-688, 1897

¹⁶ Quoted from A. W. Dox *Intracellular Enzymes of Penicillium and Aspergillus*, U. S. Dept of Agric, Bureau of Animal Industry, Bulletin 120, 70 pp, 1910

SOLUTION A		SOLUTION B	
KNO ₃	10 gram	MgSO ₄	0.5 gram
KH ₂ PO ₄	0.5 gram	K ₂ HPO ₄	0.1 gram
MgSO ₄	0.25 gram	KCl	0.5 gram
Distilled H ₂ O	100 cc	NaNO ₃	2.0 gram
		Distilled H ₂ O	1000 cc

The source of carbon was cane sugar, tannic acid or gallic acid, either alone or supplementing each other, depending upon the experiment. A 10 per cent concentration of sugar was employed, as experience has shown that the better growth is secured with this concentration than with the lower concentration. This fact is developed in a subsequent table.

Methods of inoculation In all of the fermentation experiments the method of inoculation employed was that proposed by Hasselbring.¹⁷

Methods of analysis The volumetric method of Dreaper¹⁸ was used for some experiments, but for most of the work the volumetric method proposed by Jean¹⁹ was used. Both methods have imperfections, but they are approximately accurate. In all cases analyses were checked by duplicate determinations and usually by more.

Method of washing and weighing the fungus felt For the experiments, the results of which are included in tables I and II, the method used in washing the felt free from gallic and tannic acid was as follows:

The felt was removed by means of a bent needle and floated on distilled water, the water being renewed until it gave no further coloration. In order to secure the submersed growth, the solution was poured into a cylinder and the submersed growth, which now usually floated on the surface, was then removed by needles. All of the mycelium was then placed in a crucible, which had been brought to constant weight at 105°, heated for five hours at the same temperature and then weighed. This method, as well as the use of filter paper, possesses obvious disadvantages as well as being inaccurate. For most of the work, therefore, the following method was used. The Gooch

¹⁷ H. H. Hasselbring, Carbon Assimilation of *Penicillium*, *Bot. Gazette* xlv, pp 176-193, 1908.

¹⁸ W. P. Dreaper, Estimation of Tannic and Gallic Acid, *Chem. News*, xc, pp 111-112, 1904.

¹⁹ F. Jean, Die Bestimmung des Tannins und der Gallussäure, *Chem. Centralbl.*, 1900, pp 1107-08.

filter was prepared in the usual manner, as employed in quantitative chemical analysis, and filtration was made by means of the Gooch funnel with suction. The original solution was first decanted into the Gooch crucible. The felt was then washed in the flask four or five times with distilled water at room temperature, or, if gallic acid had been precipitated, warm water was used. The washing of the felt continued until the wash water was perfectly clear. The felt was then placed in the Gooch crucible, the flask again washed and the wash water poured into the Gooch crucible. The advantages of the method consist in the rapidity of the filtration and the accuracy which results from the thorough washing, which latter is important when the culture solution is to be analyzed and absolute weight of mycelium is to be obtained. It is an especially accurate method of securing all the fungous mycelium, and by exercising a little care there is no noticeable loss of spores.

IV TOXICITY OF TANNIC ACID FOR CERTAIN FUNGI

In the literature of tannic acid fermentation only two organisms are mentioned as possessing the property of effecting this fermentation, these are *Aspergillus niger* and *Penicillium glaucum*. In order to determine whether other organisms are capable of effecting the transformation, a considerable number of filamentous fungi were carefully tested with respect to their ability to grow in tannic acid solutions.

As a nutrient medium a bean decoction was made by boiling 1 liter of laboratory preserved beans with a liter of tap water. The juice was then filtered off and diluted to 2 liters. With this decoction as a solvent, four concentrations of tannic acid were made, namely, 0.25 per cent, 2 per cent, 5 per cent and 10 per cent. Test tubes were employed as culture vessels, to each of which were added 10 cc of the solution. Small wads of filter paper were added to afford a solid substratum. The tubes were prepared in duplicate, sterilized, inoculated and kept at room temperature. They were examined at intervals and the final observations made at the end of two weeks are recorded in table A.

It is especially noteworthy that the 5 per cent permitted the growth of only one-third of the organisms, while in the 10 per cent solution only *Aspergillus flavus*, *Aspergillus niger* and *Penicillium* sp are able to grow. A separate experiment indicated that *Aspergillus oryzae* could withstand 10 per cent tannic acid.

An experiment was also made to determine if any of these organisms could utilize tannic acid as a source of carbon. Solution B with 11.6 grams of tannic acid per 100 cc of solution was used.

TABLE A

ORGANISM	CHARACTER OF GROWTH			
	0.25 per cent	2 per cent	5 per cent	10 per cent
1 <i>Penicillium brevicaulis</i>	good	good	good	
2 <i>Penicillium camemberti</i>	good	good	very slight	
3 <i>Penicillium claviforme</i>	good	good	good	
4 <i>Penicillium duclauxii</i>	good	good	none	
5 <i>Penicillium granulatum</i>	good	good	good	
6 <i>Penicillium italicum</i>	good	slight	slight	
7 <i>Penicillium lilacinum</i>	good	slight	none	
8 <i>Penicillium purpurogenum</i>	good	none	none	
9 <i>Penicillium sp</i>	good	good	good	good
10 <i>Aspergillus flavus</i>	good	good	good	good
11 <i>Aspergillus niger</i>	good	good	good	good
12 <i>Trichoderma lignorum</i>	good	none	none	
13 <i>Mucor circinelloides</i>	good	slight	none	
14 <i>Mucor rouxii</i>	good	slight	none	
15 <i>Mucor spinosus</i>	good	none	none	
16 <i>Polyporus sulphureus</i>	good	none	none	
17 <i>Polyporus resinousus</i>	good	none	none	
18 <i>Fomes megalomo</i>	no growth	none	none	
19 <i>Chaetomium sp</i>	good growth	none	none	
20 <i>Chaetostylon sp</i>	good growth	none	none	
21 <i>Stysanus sp</i>	good growth	none	none	
22 <i>Cephalothecium roseum</i>	good	none	none	
23 <i>Circinella umbellata</i>		good	none	

Erlenmeyer flasks of 150 cc capacity were employed, and in each were placed 50 cc of the culture solution. After sterilization these flasks were inoculated and maintained at room temperature. In addition to the above list of organisms the following were tested: *Aspergillus oryzae*, *Nectria ipomoeae*, *Fusarium oxysporum*, *Phycomyces nitens* and *Stilbella sp*. Of all organisms tested only *Aspergillus niger* and *Penicillium sp*²⁰ developed. Duplicate cultures of all these organisms on Chinese galls gave similar results, except in this case *Aspergillus flavus* produced a very slight growth.

VanTieghem²¹ found that both *Aspergillus niger* and *Penicillium glaucum* could withstand a saturated solution of tannic acid, and

²⁰ One other species of *Penicillium*, as indicated in the appendix, is able to develop upon a 10 per cent tannic acid solution.

²¹ Loc cit.

the fact that both develop in moistened gall nuts, which contain per dry weight 60 per cent of tannic acid, is evidence that for these two organisms the tannic acid is not toxic

Toxicity of tannic acid for Aspergillus flavus and Aspergillus oryzae

An experiment was conducted to determine the growth at various concentrations of tannic acid with and without 10 per cent cane sugar. In one case tannic acid (Merck's tested reagent) was added to solution B. In the other, tannic acid + 10 per cent sugar was used. Test-tube cultures with 15 cc of the solutions were employed. The results in general showed that these two fungi develop normally in the presence of 2.5 per cent tannic acid, but greater concentrations decrease the rate of germination and inhibit the growth. In the 15 per cent concentrations of tannic acid, after nine days, only one-third of the surface was felted. Up to 7.5 per cent concentration the entire surface was felted.

Conclusion and discussion The experiments on the toxicity of tannic acid indicate that of all the organisms tested, *Aspergillus niger* and *Penicillium sp*²² are best adapted for the tannic acid fermentation. These two organisms were, therefore, selected for more detailed investigation, though the other two organisms previously mentioned were also reserved for further study.

Since the above experiments were made, a bulletin has appeared on the toxicity of tannin by Cook and Taubenhaus²³. The majority of a large number of parasitic organisms tested by them with respect to the toxicity of tannin show retardation of growth at from 0.1 per cent to 0.8 per cent of tannin. The few saprophytic forms tested exhibit a more marked resistance. My own experiments indicate also that the saprophytic forms can withstand relatively higher concentrations of tannic acid than the parasitic forms.

²² See appendix for description of this organism

²³ M. T. Cook and J. J. Taubenhaus. The Relation of Parasitic Fungi to the Contents of the Cells of the Host Plant, Delaware Agric. Exp. Station, Bulletin 91, 77 pp., 1911

V COMPARISON OF ORGANISMS AND INFLUENCE OF TIME AND GROWTH ON FERMENTATION

Comparison of organisms Employing an infusion of gall nuts containing 10.5 per cent tannic acid, VanTieghem²⁴ found that the fermentation was completed by *Aspergillus niger* in six days and by *Penicillium glaucum* in eight days, when the temperature of incubation was 35° and the supply of oxygen was limited by keeping the flask stoppered. In the weaker concentrations, *Penicillium glaucum* fermented the tannic acid more vigorously. Since in VanTieghem's experiments the temperature was rather high and the conditions of growth approached anaerobic conditions, it was believed that a better comparison could be made by supplying the optimum conditions for the growth of each. In all the natural fermentations (in unsterilized solution) of the tannic acid, *Aspergillus niger* always developed first and then *Penicillium* sp. So marked is this succession that by exposing a nutrient solution of 10 per cent tannic acid to the air a pure culture of *Aspergillus niger* may usually be obtained. This suggests that *Aspergillus niger* may possess the greater fermentative capacity, but an experiment was required.

In the first experiment solution A and tannic acid were used, 50 cc of the solution being placed in Erlenmeyer flasks of 150 cc capacity. The flasks were sterilized for forty-five minutes at 115°, inoculated, and kept for two weeks at a temperature of from 18°-28°, the average being close to 24°. The felts were then removed, thoroughly washed, and the wash water added to the culture solution, the solution being then brought up to 500 cc volume and analyzed according to Dreaper's²⁵ method. The figures below are for *Aspergillus niger*, the averages of six cultures, while for *Penicillium* sp the averages of five cultures are given.

TABLE I

ORGANISM	TANNIC ACID IN CULTURE SOLUTION	LOSS IN TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	LOSS OF GALLIC ACID	DRY WEIGHT OF FUNGUS
	grams	grams	grams	grams	gram
<i>Aspergillus niger</i>	0.228	1.244	0.200	2.752	0.848
<i>Penicillium</i> sp	0.332	1.140	1.780	1.172	0.537
Check	1.472		2.952		

²⁴ Loc cit²⁵ Loc cit

The loss in gallic acid indicates that this substance is used by the organisms as a source of carbon, which fact agrees with the observations of VanTieghem²⁶ and Pottevin²⁷. According to VanTieghem, when growth occurs on the surface the tannic acid is utilized directly without previous conversion into gallic acid. There is no evidence for this assumption. If the tannic acid is not utilized directly, and it probably is not, then *Aspergillus niger* is a more vigorous fermentative organism than *Penicillium sp* for in the *Penicillium* culture more tannic acid remained and the decrease in gallic acid was only 39 per cent. The larger gallic acid content of the *Penicillium* culture is related to the smaller amount of growth and not to the greater practical efficiency as a fermentative organism, and this point is more apparent from later work.

In table II there are given, separately, data for four of the *Penicillium* cultures. This table emphasizes a general relation between the amount of growth and the extent of fermentation; furthermore, the disappearance of gallic acid is correlated with increased growth.

TABLE II

CULTURE NO	TANNIC ACID IN CULTURE SOLUTION	LOSS IN TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	LOSS IN GALLIC ACID	WEIGHT OF FUNGUS
	grams	grams	grams	grams	gram
Check	1 472		2 952		
1	1 216	0 156	2 396	0 556	0 295
2	0 140	1 332	2 240	0 700	0 450
3	0 152	1 320	1 760	1 192	0 550
4	0 100	1 372	1 308	1 644	0 700

Culture No. 1 seems to indicate that the tannic acid transformation is dependent upon the amount of growth, for only a small amount of tannic acid was transformed, but a greater growth during the same period in the other cultures resulted also in almost complete transformation.

Since the most economical production of gallic acid is dependent upon the amount of growth, and growth amount is a function of time, temperature, aeration, and nutrition, then these factors should be important. Growth is emphasized because with it

²⁶ *Loc cit*²⁷ *Loc cit*

the amount of the enzyme is probably correlated, at least with respect to *Penicillium sp*

Culture No 1 shows a small decrease in tannic acid and a high decrease in gallic acid. In culture No 2 the loss of tannic acid is greater than loss of gallic acid. The probable explanation is that *Penicillium sp* utilizes first the gallic acid and then transformation of the tannic acid occurs. In culture No 1 the smaller growth has been at the expense of tannic acid. It does not seem possible to demonstrate that tannic acid is not directly utilized but it seems probable that it must be converted into gallic acid.

Influence of duration and extent of growth and comparison of organisms In order to determine the yield of gallic acid at different intervals, and so to note the influence of growth and duration on the fermentation, an experiment was made as follows. Solution B was used and to it were added 7.5 grams of Merck's purified tannic acid per 100 cc of solution. The concentration of the tannic acid was such that it came within the limits, as found by Van Tieghem,²⁸ most favorable for *Penicillium glaucum*. For the investigation 50 cc of the culture solution were placed in Erlenmeyer flasks of 150 cc capacity. The cultures were sterilized for fifteen minutes at 5 pounds' pressure and then inoculated according to the method described. The cultures were kept in an incubator at 31° and, at intervals, as indicated, duplicate cultures were taken for analyses. The mycelium was removed by filtering with suction through the Gooch crucible, and it was then washed with warm water to dissolve all adhering gallic acid. The solution was then brought up to 500 cc and analyzed according to the method of Jean.²⁹ The results of the experiment are given in Table III.

In culture No 8 the amount of gallic acid decrease was 0.126 gram and the tannic acid decrease only 0.069 gram, recalling the case of No 1, table II. *Penicillium sp*, it seems, therefore utilizes the gallic acid first, and then the secretion of enzyme involves the transformation of the tannic acid. In all of the succeeding cultures the tannic acid decrease is greater than the increase of gallic acid, and this is to be accounted for, again, not by a direct utilization of tannic acid but by the fact that the tannic acid is first converted into gallic acid, and the constant increase of the

²⁸ *Loc cit*

²⁹ *Loc cit*

TABLE III
Organism, *Aspergillus niger*

CULTURE NO	DURATION days	TANNIC ACID IN CULTURE SOLUTION grams	LOSS OF TANNIC ACID grams	GALLIC ACID IN CULTURE SOLUTION grams	LOSS OR GAIN OF GALLIC ACID grams	DRY WEIGHT OF FUNGUS gram
1	4	0 750	0 853	2 584	+0 484	0 0314
2	6	1 057	0 546	2 191	+0 091	0 1568
3	8	0 252	1 351	1 814	-0 286	0 1946
4	10	0 306	1 297	1 001	-0 499	0 4688
5	12	0 160	1 443	1 460	-0 640	0 4933
6	16	0 159	1 444	1 406	-0 694	0 5137
7	28	0	1 603	0 898	-1 202	0 6345
Check		1 603		2 100		

Organism, *Penicillium sp*

8	4	1 534	0 069	1 974	-0 126	0 0157
9	6	1 330	0 273	2 191	+0 091	0 0800
10	8	1 296	0 305	1 769	-0 331	0 1458
11	10	0 750	0 853	1 669	-0 431	0 3031
12	12	0 427	1 176	1 333	-0 767	0 3880
13	16	0 597	1 006	1 530	-0 670	0 4096
14	28	0 106	1 497	1 277	-0 823	0 4284

gallic acid prevents the utilization of the gallic acid from being made manifest

If the *Aspergillus niger* and *Penicillium sp* cultures are compared, one finds that the tannic acid in the *Aspergillus* culture is transformed to the extent of 81 per cent by the tenth day, whereas in the *Penicillium* cultures this transformation, for the corresponding time, is only 53.3 per cent of the tannic acid. Moreover, by the fourth day the gallic acid had increased in culture No. 2 by nearly 0.5 gram, while in culture No. 8, for a corresponding time, there was a decrease of gallic acid. With this concentration and temperature, therefore, the *Aspergillus niger* is a more vigorous fermentative organism than *Penicillium sp*.

If now the time factor and amount of growth be examined in their relation to the gallic acid, it is found that with the above solutions and under the specified conditions, the gallic acid decreases after the sixth day and is utilized in the further metabolism of the organism.

Comparison of fermentative capacity in an infusion of gall nuts
 For the investigation an extraction of the gall nuts was made as follows 1800 grams of the Aleppo gall nuts were placed in one jar and the same quantity of Chinese nuts in another To each were added 3 liters of tap water, and the extraction was allowed to continue for five days, when the extract was filtered To each jar were added again 2 liters of water, and after one day the extracts were filtered and combined with the previous filtrates Then the two extracts from the Chinese and Aleppo galls were mixed After a second filtration the solution was ready for the culture vessels For this purpose cultures were made in the usual way, using Erlenmeyer flasks of 150 cc, each, with 50 cc of the infusion

TABLE IV

CULTURE NO	DURATION days	TANNIC ACID IN CULTURE SOLUTION grams	LOSS OF TANNIC ACID grams	GALLIC ACID IN CULTURE SOLUTION grams	GAIN OF GALLIC ACID grams	DRY WEIGHT OF FUNGUS gram
Check		1.637		1.797		

Aspergillus niger

1	4	0.545	1.092	2.752	0.955	
2	6	0.218	1.419	3.218	1.421	0.197
3	8	0	1.637	2.500	0.703	0.262
4	12	0	1.637	2.527	0.730	0.171
5	20	0.206	1.431	2.527	0.730	0.205
6	56	0	1.637	2.527	0.730	0.148

Penicillium sp

7	4	1.330	0.307	2.050	0.253	0.043
8	6	0.545	1.092	2.627	0.730	0.157
9	8	0.409	1.128	2.387	0.590	0.110
10	12	0.034	1.603	2.471	0.674	0.171
11	20	0.085	1.552	2.457	0.660	0.117
12	56	0.152	1.415	2.247	0.450	0.151

A comparison of the two organisms, as regards their fermentative capacity, shows again that *Aspergillus niger* is a more efficient organism Note especially that in four days the *Aspergillus* cultures exhibit an increase of gallic acid nearly four times as great as that in the *Penicillium* cultures, and all of the tannic acid had been converted in the former by the eighth day, while in the *Peni-*

cillium cultures only 70 per cent was converted in the same time. Not only does *Aspergillus niger* produce a more rapid fermentation, but also a greater production of gallic acid is effected, for, as indicated, the maximum yield of gallic acid in the *Aspergillus* cultures is twice that in the *Penicillium* cultures, and this in spite of the fact that a greater weight of *Aspergillus niger* is produced. Furthermore, in the *Penicillium* cultures there occurs a decrease of the gallic acid even when considerable tannic acid is still present in the culture solution.

In order to understand these differences between *Aspergillus niger* and *Penicillium sp*, the composition of the infusion must first be considered and an idea of this may be obtained from constituents of the gall nuts. In addition to tannic acid, the gall nuts contain (Gubert³⁰) gallic acid, chlorophyll, starch, gums, sugar, proteins and various inorganic salts and other compounds. A water extract of the gall nuts would contain in solution and suspension a certain amount of most of these substances, and the subsequent sterilization would probably result in transformations which would make certain of the organic compounds more available.

Aspergillus niger is an omnivorous organism in its relation to the utilization of carbon compounds. The growth of this organism for the first few days is probably at the expense of the other organic substances present, and the gallic acid, in this case, accumulates. All the facts indicate that while *Aspergillus niger* is utilizing the organic compounds other than tannic acid, it secretes the enzyme tannase (this point will be further developed in a separate paper), and consequently the transformation of the tannic acid goes on, and gallic acid accumulates. When the other organic compounds are exhausted, the gallic acid is utilized, and then the decrease begins. As shown previously, *Penicillium sp* tends to utilize the gallic acid before it transforms the tannic acid, for gallic acid is a favorable nutrient carbon compound for this organism. Furthermore, as I will show in a later paper, the presence of the other organic compounds may decrease the secretion of the enzyme tannase by *Penicillium sp*, and since the utilization of the gallic acid exceeds the formation of this substance, there results a decrease

³⁰ Quoted from H. Trimble *loc cit*

of the gallic acid despite the fact that tannic acid is present in the solution

A comparison of tables III and IV is of interest. The one experiment with a synthetic solution and the other with the infusion were conducted at the same time and under identical conditions as regards sterilization, inoculation and incubation. Moreover, the tannic acid and the gallic acid content of the culture solutions are nearly the same.

In table V, only the duration of experiment, decrease of tannic acid, the loss or gain of gallic acid and the dry weights of the fungus produced, are included.

TABLE V

DURATION days	SOLUTION B + 1.603 GRAMS TANNIC ACID + 2.100 GRAMS GALIC ACID			INFUSION OF GALL NUTS TANNIC ACID = 1.627 GRAMS GALLIC ACID = 1.797 GRAMS		
	LOSS OF TANNIC ACID	LOSS OR GAIN OF GALLIC ACID	DRY WEIGHT OF FUNGUS	LOSS OF TANNIC ACID	GAIN OF GALLIC ACID	DRY WEIGHT OF FUNGUS
	grams	gram	gram	grams	grams	gram
<i>Aspergillus niger</i>						
4	0.753	+0.484	0.031	1.092	+0.955	
6	0.546	+0.091	0.0156	1.419	+1.421	0.197
8	1.351	-0.286	0.194	1.637	+0.703	0.262
12	1.443	-0.640	0.493	1.637	+0.730	0.171
16	1.444	-0.694	0.513			
20				1.637	+0.730	0.148
<i>Penicillium sp</i>						
4	0.069	-0.126	0.015	0.307	+0.253	0.043
6	0.273	+0.091	0.080	1.092	+0.760	0.157
8	0.305	-0.331	0.145	1.128	+0.590	0.110
12	1.176	-0.767	0.188	1.603	+0.674	0.171
16	1.016	-0.670	0.4096			
20				1.415	+0.450	0.151

It is interesting to note that in the synthetic solution the gallic acid decreased after the eighth day, while in the gall nut infusion it showed at that time a marked increase, and this increase was maintained thereafter. In the synthetic solution on the eighth day there was a loss of 0.286 gram of gallic acid, while in the gall nut infusion there was a gain of 0.703 gram of gallic acid, and in

the latter the tannic acid was completely transformed. The difference in the amount of tannic acid transformed was not sufficient, however, to account for the loss of gallic acid in the synthetic solution and the marked increase in the infusion culture. Despite the greater weight of the fungus this increase of gallic acid was more in the infusion culture. A similar condition existed at the end of six days. The explanation of this point seems to be found in the fact that the infusion cultures contain various organic compounds which are utilized in place of the gallic acid, that is, elected in preference. At the end of four days probably none of the gallic acid has been assimilated. The subsequent decrease in gallic acid was due to its use after the exhaustion of more favorable organic nutrients. After the sixth day in the infusion culture there was no further growth of the organisms and no decrease in the gallic acid. The growth was less than one-half of that in the synthetic cultures, probably due to the lack of inorganic nutrients, although the presence of injurious metabolic products might also have been a factor, as the organic food supply was by no means exhausted. The conditions which obtained for the *Aspergillus niger* cultures apply also to those of *Penicillium sp*.

Another point of interest brought out by the comparison is the more rapid fermentation in the infusion than in the synthetic solution. In the *Aspergillus* cultures the tannic acid was completely transformed in the gall nut infusion by the eighth day, when the weight of the fungus was 0.262 gram. In the synthetic cultures transformation was not complete by the sixteenth day, when the weight of dry mycelium was 0.513 gram. In the *Penicillium* cultures, also, the results are comparable.

In another experiment in which a gall nut infusion was used containing 2.04 grams of tannic acid and 0.56 gram of gallic acid per 50 cc, at room temperature, there was maintained for thirty days an increase of gallic acid. Practically all of the fermentation occurred before the eleventh day, and the gallic acid was protected by the other organic substances.

Since the experiments with the infusion cultures indicated that the gallic acid was protected to a certain extent by the election of other organic substances, it was determined to try the addition of sugar to the culture solution with respect to its effect on the election of foods and hence on the yield of gallic acid.

VI INFLUENCE OF THE ADDITION OF SUGAR

Effect of 5 per cent sugar For these cultures solution A was used to which was added in the one series tannic acid alone, and to the other, tannic acid and cane sugar Sugar was added at the rate of 5 grams per 100 cc of solution, and the amount of tannic acid is indicated by the check The cultures were made in liter flasks, and 500 cc of the solution were used for each The flask and contents were sterilized, inoculated and kept in the incubator at a temperature which varied from 27°-30°C The results of the experiment given in table VI are in all cases obtained from duplicate cultures

TABLE VI

CULTURE NO	DURATION days	TANNIC ACID IN CULTURE SOLUTION grams	LOSS IN TANNIC ACID grams	GALLIC ACID IN CULTURE SOLUTION grams	GAIN OR LOSS OF GALLIC ACID grams	DRY WEIGHT OF FUNGUS grams
SERIES I <i>Solution A + tannic acid</i>						
1	10	4 568	8 193	11 095	+6 867	1 42
2	20	0 203	12 558	2 190	-2 038	3 66
3	30	0 050	12 711	0 142	-4 086	4 00
Check		12 761		4 228		
SERIES II <i>Solution A + tannic acid + cane sugar</i>						
4	10	0 913	11 848	8 761	+4 533	6 008
5	20	0 812	11 949	3 904	-0 324	10 35
6	30	0 101	12 660	0 143	-4 085	13 10
Check		12 761		4 228		

The results of the experiment seemed at first surprising Instead of getting a protective action of the sugar with respect to the gallic acid, and thereby an increased yield of gallic acid, the opposite condition seemed to result The yield of gallic acid was actually less at the end of ten days in the solution containing sugar than in the solution which lacked sugar, even though more of the tannic acid was transformed in the former At the end of twenty days more gallic acid was left in the cultures of series II than in the corresponding culture solutions of series I, and so a certain protective action of the sugar is evident At the end of thirty days practically all of the tannic acid and gallic acid had disappeared from

the culture solutions. In explanation of the seeming failure of the sugar to protect the gallic acid, the dry weights of felts produced in the corresponding cultures must be compared. The weight of fungus produced in each culture of series II was, at the end of each period, at least three times as great as the weight of the corresponding cultures of series I. This increased growth and the accompanying increased respiration were sufficient to utilize practically all of the organic nutrients supplied, usually all of the sugar and some of the gallic acid.

Effect of 10 per cent sugar. Since negative results were obtained as regards the protection of the gallic acid by 5 per cent sugar a new series of cultures was made with 10 per cent sugar in solution B to which was added the tannic acid required. On analysis the solution showed after sterilization 4.171 grams of tannic acid and 2.198 grams of gallic acid per 50 cc. The cultures were incubated at a temperature of 28°, though it dropped occasionally, owing to an imperfect thermostat, to 25° and rose likewise to 32°. The cultures were taken down at definite intervals, the weight of the felts determined, and the analyses of the culture solutions made according to the methods previously described. The results follow in table VII.

The protective action of the sugar is at once evident. Since the concentration of tannic acid here is double that in the experiments which are included in table III, and therefore a comparison of the yields of gallic acid is not possible, yet the great increase of gallic acid and the maintenance of this increase prove that the sugar has been utilized in place of the gallic acid. Here it is obviously not true that the greater the weight the less the gallic acid. Cultures Nos. 4, 5, 6 and 7 all vary in weight, yet the amount of gallic acid in each is approximately the same, and any difference may be due to the imperfect method of analysis. Even at the expiration of thirty-five days no decrease of the gallic acid was evident. It may be concluded, therefore, and further experiments prove, that the 10 per cent sugar protects the gallic acid.

While an increase of gallic acid is maintained in the *Penicillium* cultures, the increase is relatively small and is due to a slower transformation of the tannic acid, practically no transformation of tannic acid occurred until after the fourteenth day. Previous to this time the growth was entirely submersed, but afterwards fructi-

TABLE VII

CULTURE NO	DURATION days	OALIC ACID IN CULTURE SOLUTIONS grams	INCREASE OF OAL- LIC ACID grams	DRY WEIGHT OF FUNGUS gram
Check		2 198		
<i>Aspergillus niger</i>				
1	3	4 929	2 731	0 1579
2	6	5 028	2 830	0 4093
3	8	5 533	3 335	0 4135
4	14	*6 320	4 122	0 5148
5	20	*6 320	4 122	0 4470
6	25	*6 292	4 094	0 4820
7	35	*6 348	4 150	0 4986
<i>Penicillium sp</i>				
8	6	2 190	0	0 0076
9	14	2 261	0 063	0 332
10	20	3 764	1 574	0 265
11	25	3 764	1 574	lost
12	35	†3 820	1 630	0 4515
13	56	†4 101	1 911	0 5570

* Transformation of tannic acid complete

† Considerable tannic acid still left in culture solution

fication occurred. According to Malfitano³¹ the secretion of the enzymes from *Aspergillus* occurs just after fructification, and it is possible to conclude that the lack of transformation is explainable by non-secretion of the enzyme by the submersed growth. VanTieghem,³² however, found that transformation was effected when the growth was submersed, and some experiments of the writer subsequently confirm the fermentation by submersed growth. The non-transformation is probably due to the inhibiting action of the sugar on the secretion of the enzyme tannase, but not on the formation of the enzyme, for subsequent experiments have shown that with 2 per cent tannic acid and 10 per cent sugar the enzyme is formed. The inhibition of enzyme secretion by the presence of organic nutrients has been observed by Puriewitsch.³³

³¹ G Malfitano La proteolyse chez l'*Aspergillus niger*, *Ann d l'Inst Pasteur*, xiv, pp 60-81, 1900

³² Loc cit

³³ K Puriewitsch Ueber die Spaltung der Glycoside durch die Schimmelpilze, *Ber d deutsch bot Gesellsch*, xvi, pp 368-377, 1898

The transformation of salicin or arbutin was inhibited in the presence of certain amounts of glucose, cane sugar or starch. Likewise Katz³⁴ found that *Penicillium glaucum* did not secrete the enzyme diastase when, along with 0.25 per cent soluble starch, either 2 per cent glucose or 15 per cent cane sugar was offered. *Aspergillus niger* was not influenced noticeably in the secretion of the tannase by the presence of the 10 per cent cane sugar, while the *Penicillium sp.* was markedly influenced.

Another noteworthy fact in regard to the *Penicillium sp.* is the small increase of gallic acid between the twentieth and thirty-fifth day. It seems that the enzyme secreted was so very little, or of such feeble activity or destroyed, that a very limited transformation only was effected. Even at the end of fifty-six days the tannic acid was not entirely converted, and the increase of gallic acid after the end of thirty-five days was relatively small.

Influence of concentration of sugar on growth. For an explanation of the protective action of 10 per cent sugar the following table is suggestive. Each result is the average of sixteen cultures of *Aspergillus niger*.

TABLE VIII

CULTURE NO.	COMPOSITION OF NUTRIENT SOLUTION	SUGAR IN CULTURE SOLUTION	DRY WEIGHT OF FUNGUS
		per cent	grams
1	Check 50 cc sol A + trace Fe Cl ₃ + 2.5 gms sugar	5	0.779
2	50 cc sol A + trace Fe Cl ₃ + 8.55 gms sugar	17.1	1.239
3	50 cc sol A + trace Fe Cl ₃ + 17.1 gms sugar	34.2	1.513
4	50 cc sol A + trace Fe Cl ₃ + 25.65 gms sugar	51.3	1.590
5	50 cc sol A + trace Fe Cl ₃ + 37.00 gms sugar	74.0	1.308
6	50 cc sol A + trace Fe Cl ₃ + 42.50 gms sugar	85.0	1.230

The table shows that the optimum sugar content is above 5 per cent. It is possible that the increased growth in the culture solution of over 10 per cent sugar is produced not by an assimilation

³⁴ J. Katz, Die regulatorische Bildung von Diastase durch Pilze, *Jahrb f wiss Bot*, xxxi, pp 599-618, 1898.

lation of more sugar but by a stimulus, the result of high concentration. The table is offered here, however, only to indicate that with the nutrient solution used 5 per cent sugar is not sufficient for greatest growth. It throws light also upon the failure of the 5 per cent sugar to protect the gallic acid in the previous experiment.

VII ELECTION OF ORGANIC SUBSTANCES

Historical VanTieghem³⁵ stated that the glucose formed as a result of tannic acid fermentation was utilized and the gallic acid left behind. Pasteur demonstrated that *Penicillium glaucum* exhibited an election of the dextro-tartaric acid when both the dextro- and laevo-tartaric acids were present. Duclaux's³⁶ observations revealed the fact that when *Aspergillus niger* was offered salts of butyric and acetic acid in a mixture, it first used the latter and then the butyric acid. Furthermore, he proved that this was not due to the better nutrient value of acetic acid, for when the acetate was offered with the tartrate (an especially good nutrient) the acetate was utilized more rapidly. The election then was not merely a matter of relative food value.

Pfeffer³⁷ found that under certain conditions the use of glycerin by fungi may be protected by dextrose and even better by peptone, and he showed also that the relative concentration of each had an effect upon the election.

Puriewitsch³⁸ found with two organisms an election with respect to the products of amygdalin. With this substance as the source of carbon it was first transformed, then the dextrose and lastly the benzaldehyde was used. He found further that salicin was not transformed in the presence of six times its quantity of dextrose, twelve times its quantity of saccharose or fourteen to sixteen times its quantity of starch.

Election of cane sugar In order to determine definitely whether or not *Aspergillus niger* and *Penicillium sp* elect cane sugar, when it is offered together with gallic acid, two series of cultures were made. Solution B was used, to which was added in the one case the gallic acid and 10 per cent of cane sugar, the cultures being made in Erlenmeyer flasks as before. In a similar manner cultures were made in which the gallic acid alone was offered as the source of carbon. Sterilization and inoculation were made by the usual

³⁵ Loc cit

³⁶ E. DuClaux, Sur la nutrition intracellulaire, *Ann de l'Inst Pasteur*, III, pp 97-112, 1889

³⁷ W. Pfeffer, Ueber Election organischer Nahrstoffe, *Jahrb f wiss Bot*, XXVIII, pp 203-268, 1895

³⁸ Loc cit

methods, and the cultures incubated at 28° C The results obtained are included in table IX

TABLE IX

ORGANISM	DURATION days	GALLIC ACID IN CULTURE SOLUTION grams	GALLIC ACID USED grams	DRY WEIGHT OF FUNGUS grams
SERIES I <i>Solution B + 10 per cent sugar + gallic acid</i>				
Check		2 837		
<i>Aspergillus niger</i>	7	2 837	none	0 3491
<i>Penicillium sp</i>	7	2 837	none	0 1109
<i>Aspergillus niger</i>	10	2 837	none	0 4589
<i>Penicillium sp</i>	10	2 837	none	0 3676
SERIES II <i>Solution B + gallic acid</i>				
Check		2 837		
<i>Aspergillus niger</i>	7	2 429	0 337	0 1000
<i>Penicillium sp</i>	7	2 837	not detected	0 010
<i>Aspergillus niger</i>	10	1 474	1 363	0 3434
<i>Penicillium sp</i>	10	2 557	0 280	0 108

A glance at the table reveals the fact that both *Penicillium sp* and *Aspergillus niger* elect cane sugar and leave behind in the culture solution the gallic acid Cane sugar is therefore proven conclusively to protect the gallic acid It is of interest to note that the addition of the sugar permits of a more rapid and more extensive growth during the ten-day period

VIII INFLUENCE OF AERATION

Limiting supply of oxygen VanTieghem³⁹ stated that under aerobic conditions the tannic acid was utilized directly, and that the small amount of gallic acid formed was also assimilated The preceding experiments and others of the writer, not here mentioned, show that transformation of the tannic acid occurs even when all the growth is on the surface However, if sugar is not offered with the tannic acid, the increased growth may, as shown, be at the expense of the gallic acid formed If the tannic acid is

³⁹ Loc cit

offered as the only source of carbon, the yield of gallic acid is obviously dependent upon the amount of growth. Van Tieghem⁴⁰ found that 0.022 gram's weight of mycelium transformed 48.3 grams of tannic acid in ten days at 35°. The growth is greatly diminished by the absence of oxygen, consequently the oxygen supply is a factor influencing the yield of gallic acid. In order to compare the yield under aerobic and anaerobic conditions, another experiment was performed. Solution B was used, to which was added tannic acid. Into each of six Erlenmeyers of 150 cc capacity were placed 50 cc of the solution. Four of the flasks were plugged with cotton, while the other two were fitted with perforated rubber stoppers. After sterilization the perforations were plugged with pieces of glass rod and these, together with two of the flasks plugged with cotton, were inoculated with *Aspergillus niger*. The flasks fitted with the rubber stoppers contained approximately 100 cc of air and therefore about 20 cc of oxygen. The cultures were incubated at 31° and at the end of twenty-eight days and forty days analyses were made. The results follow in table X.

TABLE X

CULTURE SOLUTION	DURATION	TANNIC ACID IN CULTURE SOLUTION	LOSS OF TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	LOSS OR GAIN IN GALLIC ACID	DRY WEIGHT OF FUNGUS
	days		grams	grams	grams	gram
Anaerobic (limited)	40	0	1.603	3.286	+1.186	0.158
Aerobic	28	0	1.603	0.898	-1.202	0.634
Check		1.603		2.100		

It is at once evident that the inhibition of growth due to deficiency of oxygen is favorable to a good yield of gallic acid. No doubt if the culture in limited oxygen supply had been analyzed sooner a greater yield of gallic acid could have been obtained. With respect to the condition in aerobic cultures it may be stated that cultures, identical with those above, showed on the fourth day a gain of 0.484 gram of gallic acid, though only half of the tannic acid had been transformed, and the weight of the mycelium produced was 0.0314 gram.

Comparison of methods In order to determine more definitely the yield of the gallic acid under conditions in which the supply of

⁴⁰ Loc. cit.

oxygen varied, as well as to compare the yields under these conditions with that obtained when the most favorable conditions were offered, as by the addition of sugar, the following experiment was made

SERIES I Solution B + tannic acid, flask plugged with cotton and aerobic conditions maintained

SERIES II Solution B + tannic acid + 10 per cent cane sugar, otherwise like the above

SERIES III Solution B + tannic acid, flasks stoppered with rubber stoppers and containing therefore only 75 cc of air or approximately 15 cc of oxygen

SERIES IV Solution B + tannic acid, flasks stoppered with perforated rubber stoppers, fitted with glass and rubber tubing and clamps The air was replaced by passing a stream of nitrogen (oxygen-free air) through the flasks for a period of five minutes after inoculation had been made

All the inoculations were made with spores of *Aspergillus niger*, according to the method described by Hasselbring ⁴¹ The temperature of incubation varied from 30°-35° Erlenmeyer flasks of 125 cc capacity were employed. The results obtained are included in table XI

TABLE XI
Aspergillus niger Duration, ten days

SERIES	ATMOSPHERE OF GROWTH CHAMBER	TANNIC ACID IN CULTURE SOLUTION	LOSS OF TANNIC ACID	GALLIC ACID IN CULTURE SOLUTION	GAIN IN GALLIC ACID	DRY WEIGHT OF FUNGUS
		grams	grams	grams	grams	gram
Check		4 433		3 089		
I	Unlimited air supply	0 409	4 024	5 618	2 529	0 3166
II	Unlimited air supply	0 683	3 760	6 515	3 426	0 3341
III	75 cc air	1 023	3 410	6 151	3 062	0 0114
IV	Nitrogen	1 707	2 726	5 420	2 331	0 0013

In comparing the rate of tannic acid transformation under the different conditions it is found that in the order of rapidity of transformation they are series I to series IV. The yield of gallic acid was greatest in series II, the addition of cane sugar in this case protecting the gallic acid, somewhat less in series III, still less in series I, where the mycelium was abundant, despite the fact that it led in the amount of tannic acid transformed, and last in series IV. It is noteworthy that the amount of mycelium pro-

⁴¹ Loc cit

duced in this last was only 1.3 mgm, yet sufficient of the enzyme was liberated to transform a quantity of tannic acid more than 2000 times the weight of the mycelium produced.

From an economic standpoint the method of series I is wasteful of gallic acid, as the organism utilizes much of this substance in its metabolism. Series II has an advantage over series III, and series III over series IV, only in the rapidity of the transformation. The small amounts of growth in series III and IV require such a slight amount of gallic acid in their metabolism that the yield of gallic acid in those series would finally be practically equal to that obtained in series II, to the cultures of which sugar had been added. This fact is borne out by the larger amounts of tannic acid left in the culture solutions of series II and series III.

IX. SUMMARY

1 Tannic acid is toxic to a large number of fungi at relatively low concentrations.

2 *Aspergillus niger* is a more vigorous fermentative organism than *Penicillium* sp.

3 The fermentation was found to be more rapid in the gall nut infusion than in the synthetic solution in which tannic acid was the only source of carbon. The presence of other organic compounds in the gall nut infusion protected to a certain extent the gallic acid.

4 The addition of 5 per cent sugar did not protect the gallic acid but simply increased the growth. The addition of 10 per cent sugar protected the gallic acid entirely.

5 When gallic acid and cane sugar to the extent of 5.5 per cent and 10 per cent, respectively, were offered together, the cane sugar was elected and the gallic acid left in the culture solution.

6 Fermentation can take place under anaerobic conditions, and 1 mgm of mycelium is sufficient to effect the transformation of 2.706 grams of tannic acid in ten days.

7 In an approximately 15 per cent solution of tannic acid, fermentation was most rapid when the tannic acid alone served as the source of carbon, and when aerobic conditions were maintained, yet the method of fermentation is wasteful from the standpoint of an economical yield of gallic acid.

8 The economical methods are (a) those in which growth occurs under aerobic conditions and the tannic acid is supplemented by cane sugar, or (b) those in which, with tannic acid alone, the supply of oxygen is limited to a small amount

9 The presence of 10 per cent cane sugar does not inhibit the secretion of the enzyme tannase by *Aspergillus niger*, but it does seem to inhibit to some extent the secretion of the tannase by *Penicillium sp*

10 The enzyme is secreted into the culture solution by submerged mycelium as well as by surface growth. There is no evidence that tannic acid is used directly, but the evidence seems to indicate that tannic acid is first transformed into gallic acid and the gallic acid then utilized

APPENDIX

Investigators previously occupied with tannic acid fermentation usually employed *Aspergillus niger* together with a species of *Penicillium* which they have designated *Penicillium glaucum*. As has been pointed out by Thom⁴² the name *Penicillium glaucum* has in the past been applied to so many different species that the only idea conveyed by its use is a general concept of the genus. VanTieghem⁴³ applied the name to denote the species of *Penicillium* which he isolated from gall nuts, and it is probable that other students of tannic acid fermentation used the same organism.

In the work of the writer a trial of a number of organisms was made and the *Penicillium* employed was secured from a culture labeled *Penicillium olivaceum*. That identification was incorrect, and then it was believed that the organism might be the one which develops on the gall nuts, but this also proved erroneous, as is indicated subsequently.

In attempting to determine the *Penicillium sp* used in these experiments it was found that the organism did not correspond to any of the species described by Thom.⁴⁴ Instead, however, of withholding publication of these investigations until the organism should be definitely determined, it was thought best to present here a brief description and certain cultural characteristics of the organism. It is, furthermore, the intention of the writer to make a study of the relation of the various species of *Penicillium* to tannic acid fermentation, and it is hoped by that time to have determined definitely the two species of *Penicillium* which are now known to develop in a 10 per cent solution of tannic acid.

The *Penicillium sp* used in these experiments possesses only a single whorl of unbranched conidia-bearing cells (sterigmata), and might therefore

⁴² Charles Thom, Cultural Studies of *Penicillium*, Bureau of Animal Industry, Bulletin 118, pp. 107, 1910

⁴³ *Loc cit*

⁴⁴ *Loc cit*

he grouped with the genus *Citromyces* as founded by Wehmer, but Thom⁴⁵ does not consider this a valid basis for differentiation of genera, and prefers to include this form of conidiophore under the genus *Penicillium*. This latter concept is here followed.

Description of organism Colonies on 15 per cent gelatin are, when young, of a faint green color which changes to an otter brown. On 15 per cent gelatin + 3 per cent sugar the olive green changes to ashy gray and then to greenish black. On bean agar the color is at first bluish green, and changes to dark olive green, and finally to a grayish color. The surface is velvety. The conidiophores arise vertically from the substratum and in length vary from 100μ to 700μ . The fructification averages 90μ in length (it may be 200μ), and its width is approximately 15μ . The conidiferous cells average 10μ in length, the conidia are spherical, and 3μ in diameter. A single whorl of simple conidia-bearing cells only is present as is represented by figures 1 and 2.



FIG 1

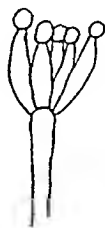


FIG 2

Conidial fructification showing simple conidiferous cells ($\times 600$)

Cultural character At room temperature 15 per cent gelatin is liquified in six days with the production of a strong ammoniacal odor, in the presence of 3 per cent sugar the total liquefaction is retarded a day or more. The presence of bean juice still longer delays the liquefaction of the gelatin. On 15 per cent gelatin + 3 per cent sugar, the lower surface is colored yellowish to reddish brown, when grown in solution B + 10 per cent cane sugar the lower surface of the mycelial felt may be of a salmon color. Fruiting on such a solution at room temperature usually requires eight days. This organism possesses the ability to ferment tannic acid and with 10 per cent tannic acid in solution B at a temperature of 30°C , gallic acid may be precipitated in about seven days.

Gall nut *Penicillium* Only one other species of *Penicillium* has so far been found to grow on 10 per cent tannic acid, and this is the one isolated from the gall nuts. It grows more slowly in 10 per cent tannic acid. In the presence of sugar it produces an intensely red color in the substratum and is a slow liquefier of gelatin. It differs from the other also in possessing more than one whorl of conidiferous cells, and has other distinguishing features. It appears to be *Penicillium rugulosum*.

TANNIC ACID FERMENTATION II

EFFECT OF NUTRITION ON THE PRODUCTION OF THE ENZYME TANNASE

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I PREFACE

In an investigation upon tannic acid fermentation reported in the previous paper, it was found that when cane sugar and tannic acid are offered simultaneously to either *Aspergillus niger* or *Penicillium sp*, the sugar is utilized as the source of carbon while the tannic acid is fermented, gallic acid resulting. Some of the results indicated that the rate of fermentation was influenced by the concentration of the sugar. It was deemed important, therefore, to determine if varying the relative amounts of tannic acid and sugar in the nutrient solution has an influence upon the amount of the enzyme tannase produced in the fungus thus grown. Since tannic acid is probably not commonly utilized in nature by *Aspergillus niger* and *Penicillium sp* as a source of carbon, experiments were also made to determine if the enzyme is produced when the fungus is cultivated on nutrient solution lacking tannic acid.

The writer wishes to acknowledge his indebtedness to Prof. B. M. Duggar for assistance received during the course of the investigation.

II INTRODUCTION

Regulatory production of enzymes. Historical. A number of investigations have been made on the regulatory formation of the enzymes, but for the most part conclusive investigations are lacking. Experimenting with two species of bacillus, Brunton and MacFayden¹ found that when culti-

¹ T. Lauder Brunton and A. MacFayden. The Ferment Action of Bacteria, *Proc. Roy. Soc.*, xlv, B, pp. 543-553, 1899.

vated on starch paste these developed the enzyme diastase, but if the starch were replaced by meat extract no diastase was formed. Fermi² found that of ten bacterial organisms, which developed the tryptic ferment in the presence of peptones or albumen, none developed the enzyme on a sugar-containing nutrient solution. Wortman's³ observations established the fact that the addition of tartaric acid prevented the formation of diastase in bacteria which inhabited decaying potatoes. Fermi and Montesano's⁴ investigations indicated that the presence of sugar is not absolutely necessary for the formation of the enzyme invertase. Various other investigators have studied the influence of nutrition on the formation of enzymes in bacteria.

Dubourg⁵ stated that a yeast which did not normally possess the inverting enzyme was capable of developing it by proper cultivation. As a culture solution in the latter case, yeast water was used, to which was added 5 per cent cane sugar and 5 per cent grape sugar. The yeast, after cultivation, was thoroughly washed and then transferred to a cane sugar solution. In the latter inversion occurred. The form of yeast was not an identified strain. He reported that he was also able to develop the enzyme which fermented galactose by similar methods.

Klöcker,⁶ employing the methods of Dubourg, was unable to develop invertase in *Saccharomyces apiculatus* or maltase in *Saccharomyces marxianus* which organisms do not normally possess these enzymes. The ability to form specific enzymes, according to Klöcker, is therefore a constant character of the yeast organism.

Recently Harden and Norris⁷ "have trained" the yeast *Saccharomyces Carlsberg I* to ferment galactose by cultivating the yeast on hydrolyzed lactose in yeast water to which was added 0.15 per cent of monobasic potassium phosphate. Normally galactose is not fermentable by the above mentioned yeast. According to Kohl⁸ the chemical nature of the solution and the aeration of the culture influence the amount or activity of the enzyme formed in the yeast organism, while the temperature of storage of the yeast also markedly affects the enzyme content.

¹ Claudio Fermi Weitere Untersuchungen über tryptischen Enzyme der Mikroorganismen, *Centralbl f Bact*, x, pp 401-403, 1891

² J Wortman Untersuchungen über das diastatische Ferment der Bacterien, *Zeitschr f physiol Chem*, vi, p 287, 1882

³ C Fermi and C Montesano Die von den Mikrohen bedingte Inversion des Rohrzuckers, *Centralbl f Bact*, i, Abt II, pp 482-87, 542-56

⁴ E Dubourg De la fermentation des saccharides, *Compt rend de l'Acad des Sci*, cxviii, pp 440-42

⁵ Alh Klöcker Ist die Enzymehildung bei der Alkoholgärungspilzen ein werthvolles Artmerkmal?, *Centralbl f Bact*, vi, Abt II, pp 241-45, 1900

⁷ A Harden and R V Norris The Fermentation of Galactose by Yeast and Yeast Juice, *Proc Roy Soc*, lxxxii, B, pp 645-49, 1910

⁸ F G Kohl Die Hefpilze, 1903, pp 79-81

Büsgen⁹ showed that *Aspergillus oryzae* on bouillon, as well as in a sugar-containing solution, formed the enzyme diastase. According to Pfeffer,¹⁰ *Penicillium glaucum* did not secrete diastase in the presence of 10 per cent sugar and, even when only 1.5 per cent sugar was present, the starch was only slightly attacked. *Aspergillus niger* behaved differently, producing diastase even in the presence of 30 per cent cane sugar. Employing a nutrient solution containing 0.25 per cent of soluble starch Katz¹¹ found that starch was saccharified by *Penicillium glaucum*. The addition of 2 per cent grape sugar or 1.5 per cent cane sugar prevented the formation of the diastase. An addition of 1.5 per cent cane sugar depressed the formation of the diastase, while an addition of 0.05 per cent had no effect. Lactose and maltose in a 3 per cent concentration decreased the rate of starch transformation, while a 10 per cent concentration still further depressed the formation of diastase. A 4 per cent addition of erythro-dextrin had no effect whatsoever in protecting the starch. Neither did a 10 per cent addition of quinic acid, 4 per cent glycerin or 2 per cent potassium tartrate have any effect upon the secretion of the diastase. The addition of peptone to the solution increased the secretion of the diastase. With *Aspergillus niger* the growth on starch nutrient solution was slow, and five days were required for the transformation of the starch. The addition of 1.5 per cent cane sugar decreased the time to two days, 15 per cent sugar increased the time of transformation by one day and 30 per cent sugar increased the time by two days. *Bacterium megatherium* behaved much the same as *Penicillium glaucum*.

Dox¹² has shown that the carbohydrate-splitting enzymes, amylase, inulase, raffinase, sucrase, maltase and lactase are formed in *Penicillium camemberti*, regardless of the carbohydrate which has served as the source of carbon in the nutrient solution. The amount of the particular enzyme could be increased, however, by cultivating the organism on the corresponding carbohydrate. Likewise, other enzymes are formed independently of the presence in the nutrient solution of the corresponding substance on which the enzyme acts.

According to Went,¹³ the ten enzymes which he investigated in *Monilia sitophila* could be divided into three groups according to the influence of nutrition on their formation. The first group includes those which are formed in slight amounts regardless of the nutrition, the second group in-

⁹ M. Büsgen, *Aspergillus oryzae*, *Ber. d. deutsch. bot. Gesellsch.*, III, pp. 66-77, 1885.

¹⁰ Quoted from R. Green, *The Soluble Ferments and Fermentation*, p. 32.

¹¹ J. Katz, Die regulatorische Bildung von Diastase durch Pilze, *Jahrb. f. wiss. Bot.*, XXVI, pp. 599-618, 1898.

¹² A. W. Dox, The intracellular Enzymes of *Penicillium* and *Aspergillus*, U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 120, 70 pp., 1910.

¹³ F. C. Went, Ueber den Einfluss der Nahrung auf die Enzyymbildung durch *Monilia sitophila* (Mont.), Sacc., *Jahrb. f. wiss. Bot.*, XXVI, pp. 611-664, 1901.

cludes the enzymes which are formed only under several different forms of nutrition, while the third group includes such enzymes as are formed only when the substance on which the enzyme acts is present in the culture solution

Butkewitsch¹⁴ also has shown that nutrition has an influence upon the secretion of the gelatin-dissolving enzyme by *Aspergillus* and *Penicillium*

III METHODS OF EXPERIMENTATION

In all of the experiments upon the influence of nutrition on the production of the enzyme the organisms were cultivated in a synthetic nutrient solution, the inorganic composition being that of Czapek's¹⁵ formula Throughout this paper the solution has been designated for convenience solution B and is as follows

Magnesium sulphate (cryst)	0 5 gram
Dibasic potassium phosphate	1 0 gram
Potassium chloride	0 5 gram
Sodium nitrate	2 0 grams
Distilled water	1000 cc

All cultures were grown at a temperature of 28° or 30° The fungus felt, when formed and before spore production, was removed from the nutrient solution, as it has been shown by Malfitano¹⁶ that the most abundant secretion of enzymes into the culture solution takes place just after spore formation The felt after removal was treated according to Albert and Buchner's method for the preparation of "Acetondauerhefe," as described by Dox,¹⁷ though in the method here employed the felt was not run through a hashing machine After the mycelium was dry, it was pulverized in a mortar and then placed in a vial until its enzymatic activity was to be determined

For determining the presence of the enzyme or the relative

¹⁴ W Butkewitsch Umwandlung der Eiweisstoffe durch die niederen Pilze im Zusammenhange mit einer Bedingungen ihrer Entwicklung, *Jahrb f wiss Bot*, LVIII, pp 147-240, 1903

¹⁵ Quoted from A W Dox The Intracellular Enzymes of *Penicillium* and *Aspergillus*, U S Dept of Agric, Bureau of Animal Industry, *Bulletin* 120, 70 pp, 1910

¹⁶ G Malfitano La proteolyse chez l'*Aspergillus niger*, *Ann de l'Inst Pasteur*, XIV, pp 60-81, 1900

¹⁷ A W Dox The Intracellular Enzymes of *Penicillium* and *Aspergillus*, *loc cit*

amount of it present, the procedure was as follows. Either a 0.5 per cent, a 0.75 per cent or a 1.0 per cent solution of tannic acid was employed. To the flasks containing the solution were then added equal weights of the pulverized mycelium, the enzymatic activity of which was to be determined. There was also added, as an antiseptic, 2 per cent toluene. The flasks containing the solution and mycelium powder, being tightly stoppered, were then incubated for a definite period and then the solution analyzed for gallic acid according to Jean's¹⁸ method. The relative increase in the gallic acid is taken as a measure of the amount of tannase present.

IV INFLUENCE OF CONCENTRATION OF SUGAR AND TANNIC ACID ON PRODUCTION OF TANNASE

Influence of concentration of tannic acid on the amount of enzyme produced. It was found in certain experiments,¹⁹ in which 10 per cent sugar plus tannic acid had been added to the nutrient solution, that the addition of sugar could not prevent the secretion of the enzyme tannase by *Aspergillus niger*, but the secretion by *Penicillium sp* was markedly decreased. In order to determine the minimum concentration of tannic acid which would stimulate the formation of the enzyme tannase and also what influence the concentration of tannic acid would have upon the amount of tannase produced within the organism, the following experiment was made. Solution B was used, to which was added 10 per cent cane sugar. Liter flasks were employed, and to each were added 500 cc of the solution. The flasks were plugged and sterilized, and when cool the tannic acid was added to each in varying amounts, as is indicated in the table. For inoculation the method described by Hasselbring²⁰ was employed, though 1 cc of the spore-containing water was used instead of a single drop. These cultures were incubated at 28°C, and the felt was then removed and treated according to the method described. In determining the enzymatic activity of the mycelium powder 0.3

¹⁸ F. Jean. Die Bestimmung des Tannins und der Gallussäure, *Chem Centralbl*, 1900, pp. 1107-08.

¹⁹ L. Knudson. Tannic acid Fermentation I, this *Journal*, xiv, p. 159, 1913.

²⁰ H. H. Hasselbring. Carbon Assimilation of *Penicillium*, *Bot Gazette*, xlv, pp. 176-193, 1908.

gram of the dried powder was added to a flask containing the tannic acid solution. Each flask contained 75 cc of an approximately 0.9 per cent solution to which was added, as an antiseptic, 1 cc of toluene. The results after one week's incubation at 34° are given in table I. The solution contained at the beginning of incubation 0.555 gram tannic acid and 0.327 gram gallic acid.

TABLE I

*Effect of concentration of tannic acid on production of enzyme tannase, using nutrient solution B + 10 per cent sugar + tannic acid. Period of incubation, 7 days.**

	AMOUNT OF TANNIC ACID ADDED	GAIN IN GALLIC ACID		AMOUNT OF TANNIC ACID ADDED	GAIN IN GALLIC ACID
	per cent	gram		per cent	gram
<i>Aspergillus niger</i>	0.00	0	<i>Penicillium</i> sp.	0.01	0
	0.01	0		0.10	0.058
	0.10	0.059		0.40	0.075
	0.80	0.223		0.80	0.065
	1.00	0.242		1.00	0.108
	2.00	0.388		2.00	0.159
	4.00	0.515		4.00	0.293
	10.00	0.515			
	10.00	0.525			
		No sugar			

* The actual concentration of each culture was approximately only two-thirds of the figures given, the other one-third consisting of gallic acid.

It is evident from the preceding table that there is a regulatory formation of the enzyme. There is a progressive increase in the amount of tannase with increase of tannic acid in the culture solution. It is noteworthy that no tannase was produced when growth took place in a nutrient solution which lacked tannic acid. It is somewhat remarkable that the formation of enzyme could be stimulated by 0.1 per cent of tannic acid (actually about 0.066 per cent) when there was present at the same time cane sugar in an amount more than one hundred times as great as the tannic acid. The stimulation by this small amount of tannic acid is even more surprising when previous experiments²¹ are recalled in which the gallic acid formed from the tannic acid was protected by the cane sugar, no determinable amount of the gallic acid being assimilated.

²¹ L. Knudson *loc cit*

Why the increase in concentration of tannic acid should increase the amount of tannase is difficult to explain. The amount removed by the organism from the solution is undeterminable with the present method of analysis.

Is the stimulation produced within the cell, or is it caused by contact of the tannic acid with the plasma membrane? Tannic acid precipitates albuminous substances, and it might be possible that it reacts in this manner with the plasma membrane and this precipitation might be the stimulus for the production of the enzyme. This explanation would not, however, include the stimulation to production of the tannase by gallic acid.

A suggestive explanation for the increase of the tannase with increased concentration of the tannic acid is afforded by the work of Katz.²² In his experiments Katz found that diastase could be precipitated by tannic acid and rendered inactive, though when freed from the tannic acid by washing with alcohol it becomes active. Bearing in mind this precipitation by tannic acid and working on the hypothesis that if the diastase formed and secreted into the culture solution were removed from solution more diastase would be formed, he added tannic acid to the culture solution, assuming that in this way there should be an increase in the quantity of the diastase formed. In his experiment Katz actually found that the total quantity of enzyme formed (that secreted into the nutrient solution and that present in the fungus) was greater in the culture which contained 0.5 per cent tannic acid than in the control, the ratio of the diastatic activity being 143 to 100. The results seem to confirm his hypothesis, but there are a number of factors which suggest another explanation of the results obtained. In some previous experiments²³ of the writer it is noted that in the presence of 10 per cent sugar the tannic acid was fermented, and in table I, here reported, it is clear that even in the presence of only 0.1 per cent tannic acid the enzyme tannase is formed and in all probability secreted. The tannic acid of the culture solution would, therefore, be fermented and rendered inactive as regards its capacity to precipitate the diastase liberated, and this condition doubtless occurred in the experiment of Katz.

²² J. Katz, *Die regulatorische Bildung von Diastase durch Pilze*, *Jahrb f wiss Bot*, xxxi, pp 599-618, 1898.

²³ *Loc cit*

While a temporary precipitate of the diastase and tannic acid may exist, it is more reasonable to assume that the increase of diastase must be ascribed to some other cause. Increased growth may possibly occur, as a result of the addition of tannic acid, and with this may be correlated an increase of diastase.

A comparison of *Aspergillus niger* and *Penicillium sp* in the formation of the enzyme tannase is of interest because it affords a partial explanation of the relatively slower transformation of the tannic acid by *Penicillium sp*²⁴. In both cases the amount of tannase produced in a 0.1 per cent concentration of tannic acid is the same. At 2 per cent concentration the amount of gallic acid, resulting from the action of the *Aspergillus* powder, was 3.88 times the transformation effected by the *Penicillium* powder.

In the preceding experiment no attempt was made to determine the amount of enzyme secreted into the nutrient solution, and an experiment was required to determine this point as well as to verify the preceding results. The methods of experimentation were essentially the same as in the previous experiment except that 250 cc Erlenmeyer flasks were employed with 100 cc of the nutri-

TABLE II

Aspergillus niger

Effect of concentration of tannic acid on the production of tannase, using solution B + 10 per cent cane sugar + tannic acid. Average period of incubation for mycelial powder, sixty-four hours, for enzyme excreted, ninety hours.

AMOUNT TANNIC ACID ADDED	WEIGHT OF FUNGUS AVERAGE OF 2 CULTURES	ENZYME OF POW- DERED MYCELIUM AVERAGE OF 2 INCREASE OF GAL- LIC ACID	ENZYME EXCRETED AVERAGE OF 2 INCREASE OF GAL- LIC ACID	TOTAL INCREASE OF GALLIC ACID
per cent	gram	gram	gram	gram
0	0.083	0	0	0
0.5	0.102	0.013	0	0.013
0.10	0.111	0.011	0	0.011
0.50	0.149	0.064	0.008	0.072
1.0	0.290	0.084	0.033	0.117
2.0	0.323	0.090	0.041	0.131
4.0	0.207	0.103	0.033	0.136
8.0	0.034	0.157	0	0.157

ent solution The tannic acid in all cases was added after the culture solutions had been sterilized The fungus mat was removed and treated as previously described, and for testing its tannase content 0.05 gram of the mycelium powder was added to 50 cc of 1 per cent tannic acid solution, containing as an anti-septic 1 per cent toluene The experiments were all made in duplicate and one series of the solutions was incubated for forty-eight hours and the other for eighty hours

After the fungus felts had been removed from the culture solution and the liquid filtered, alcohol was added to precipitate any enzymes present, the precipitate being collected on filter paper The filter paper with whatever precipitates accompanying were then added to flasks containing the tannic acid solution After ninety-six hours' incubation these solutions were analyzed for gallic acid The results obtained are given in table II

The results obtained verify the figures of the preceding table With an increase in concentration of tannic acid there is a corresponding increase in the amount of enzyme produced The excretion of enzyme into the culture solutions was evident only in the cultures having a relatively high percentage of tannic acid and at most the excretion was small

Influence of concentration of sugar Since in the preceding experiments the amount of tannase produced per unit weight of the fungus varies with the concentration of the tannic acid, it seemed desirable to determine the effect of maintaining constant the tannic acid concentration while varying the concentration of the cane sugar In the first experiment duplicate cultures of *Aspergillus niger* were made in liter flasks containing 300 cc of solution B + 2 per cent tannic acid + varying amounts of sugar The mycelial mats formed were removed, treated as before described and then tested for tannase activity In measuring the activity of the tannase of each culture 100 cc of a 0.5 per cent tannic acid solution were employed to which was added 1 per cent toluene as an anti-septic To each flask was added 0.2 gram of the powdered mycelium Incubation was given at 34°, and at the end of ninety hours the solutions were analyzed for gallic acid In table III are given the results of the experiment The results are the averages of the two tests

It is here clearly shown that with increased concentration of

TABLE III

Aspergillus niger

Effect of concentration of sugar on production of tannase, using solution B + 2 per cent tannic acid + cane sugar Period of incubation, ninety hours

AMOUNT OF CANE SUGAR ADDED	INCREASE OF GALLIC ACID	AMOUNT OF CANE SUGAR ADDED	INCREASE OF GALLIC ACID
<i>per cent</i>	<i>gram</i>	<i>per cent</i>	<i>gram</i>
0	0 224	+12	0 166
+1	0 206	+16	0 134
+2	0 206	+24	0 130
+8	0 179		

sugar, the tannic acid being maintained at a constant figure, there is a decrease in the amount of the enzyme produced

In order to verify the above results another experiment was made along substantially the same lines with *Aspergillus niger*. To test the tannase activity of the dry mycelium 100 cc of a 0.5 per cent tannic acid solution were used to which was added 1 per cent toluene. In each case 0.05 gram of the powdered mycelium was employed. The cultures were made in duplicate and the determinations likewise. One set was incubated at 24°C for four days and the second set at the same temperature for six days before analyses were made for gallic acid. In the following table there is given the composition of the nutrient solutions used and the increase in the gallic acid resulting, which increase of course is a measure of the tannase activity of the various cultures.

TABLE IV

Aspergillus niger

Effect of concentration of sugar on production of tannase, using solution B + 2 per cent tannic acid + cane sugar

AMOUNT OF SUGAR ADDED	DRY WEIGHT OF FUNGUS AVERAGE OF 2	INCREASE IN GALLIC ACID 4 DAYS INCUBATION	INCREASE IN GALLIC ACID 6 DAYS INCUBATION	AVERAGE INCREASE
<i>per cent</i>	<i>gram</i>	<i>gram</i>	<i>gram</i>	<i>gram</i>
32	0 158	0 179	0 179	0 179
16	0 083	0 270	0 278	0 274
12	0 074	0 233	0 270	0 251
8	0 101	0 270	0 278	0 274
4	0 093	0 270		0 270
2	0 065	0 270	0 296	0 283
0.5	0 074	0 303	0 332	0 317
0	0 035	0 303	0 368	0 335

The results confirm the evidence of the preceding experiment, though the differences due to sugar concentration are not so marked. This may have been due to the longer period of incubation.

V INFLUENCE OF NUTRITION

Since Dox has found in *Penicillium camemberti* that the various enzymes are produced irrespective of the nutrition of the fungus, an experiment was made to determine definitely if the enzyme tannase could be produced in two *Penicillium* species when tannic acid is withheld from the culture solution. *Penicillium sp.*, which I have previously described,²⁵ and *Penicillium rugulosum* were employed in the experiment. They were cultivated in 100 cc. of a nutrient solution which was composed on the one hand of solution B + 5 per cent sugar and, on the other, solution B + 5 per cent sugar + 2 per cent tannic acid. The mycelial felts, as before, were removed just before spore formation, treated as previously described and pulverized. For determining the presence of the enzyme tannase 100 cc. of a 1 per cent tannic acid solution were used to which was added 2 per cent toluene as an antiseptic and 0.1 gram of the pulverized mycelium. The solutions were incubated for twenty-eight days at a temperature of 33°. They were then analyzed for increase in gallic acid. All cultures and determinations were made in triplicate. As is evident from the table tannase was formed only in the presence of tannic acid.

TABLE V

ORGANISM	COMPOSITION OF SOLUTION	GALLIC ACID PRESENT	GAIN
<i>Penicillium sp.</i>	Solution B + 5 per cent sugar	0.287	0
<i>Penicillium rugulosum</i>	Solution B + 5 per cent sugar	0.287	0
<i>Penicillium sp.</i>	Solution B + 5 per cent sugar + 2 per cent tannic acid	0.822	0.535
<i>Penicillium rugulosum</i>	Solution B + 5 per cent sugar + 2 per cent tannic acid	0.822	0.535
Check		0.287	0

Experiments were next made to determine the influence of replacing the sugar by other compounds and of adding to the nutrient solution various reagents. For this experiment solution

²⁵ Loc. cit.

A²⁶ was used, and as culture vessels liter flasks were employed. Into each flask were placed 365 cc of the solution, and to it were added the sugar and other reagent, or the sugar was omitted and some other carbon compound substituted for it. The solutions were sterilized, inoculated and incubated at a temperature of 28°C. The felt was removed just before spore production and treated in the manner previously described.

To determine the presence of tannase, 0.3 gram of the powdered, dried mycelium was introduced into 100 cc of a 0.75 per cent solution of tannic acid, to which had been added as an antiseptic 2 cc of chloroform. After incubation at 31° for one week, the solutions were analyzed for gallic acid. The results obtained are given in table VI.

TABLE VI
Penicillium sp

COMPOSITION OF NUTRIENT SOLUTION	GALLIC ACID PRESENT	GALLIC ACID INCREASE
Solution A + 25 grams cane sugar	0.202	0
Solution A + 25 grams cane sugar 5 cc $\frac{N}{10}$ HCl	0.202	0
Solution A + 15 grams corn starch	0.208	0
Solution A + 25 grams glycerin	0.202	0
Solution A + 25 grams gallic acid	0.370	0.128
Solution A + 25 grams tannic acid	0.713	0.511

The control contained 0.202 gram gallic acid at the beginning and at the end of the incubation.

The gallic acid stimulated the formation of the enzyme only one-fourth as much as did the tannic acid. Slight acidity had no effect in stimulating the production of the enzyme. Glycerin and starch, both of which are relatively poor food compounds, were supplied, and if enzymes are stimulated to formation by conditions approaching starvation, as suggested by Wortman,²⁷ then the tannase should have developed, but the results were negative.

An experiment similar to that above was made with *Aspergillus niger*, 400 cc of solution being used and the methods of experimentation the same as before. The results obtained are as follows:

²⁶ KH₂PO₄, 0.5 gram, KNO₃, 1 gram, MgSO₄, 0.25 gram, Distilled water, 100 cc.

²⁷ *Loc. cit*

TABLE VII
Aspergillus niger

COMPOSITION OF NUTRIMENT SOLUTION	OALLIC ACID PRESENT	OALLIC ACID INCREASE
Solution A + 25 grams sugar	0 209	0
Solution A + 25 grams sugar + 5 cc $\frac{N}{16}$ HCl	0 202	0
Solution A + 25 grams sugar + 5 grams gallic acid	0 202	0
Solution A + 25 grams sugar + 2 grams resorcin	0 190	0
Solution A + 25 grams sugar + 1 grams hydroquinone	0 202	0
Solution A + 25 grams sugar + 5 grams peptone	0 202	0
Solution A + 25 grams sugar tannic acid	0 691	0 489

From the above table, it is evident that in *Aspergillus niger* there is a very marked regulatory formation of the enzyme Peptone, which stimulates the secretion of diastase, according to Katz²⁸ has no influence in stimulating the formation of tannase. Gallic acid of the strength used had no effect, which result is surprising in view of the results indicated in table VI and of the further fact that Pottevin²⁹ found the enzyme tannase to be formed in *Aspergillus niger* when the organism is cultivated in Raulin's solution with the sugar replaced by gallic acid. Since pyrogallol is a decomposition product of tannic acid, it was thought that perhaps it might stimulate the formation of the enzyme, but the amounts used proved toxic in this particular experiment. Resorcin and hydroquinone were both employed because of their constitutional similarity to pyrogallol, both being dihydroxybenzenes, while the pyrogallol is a trihydroxybenzene. Negative results, however, were obtained.

In a similar manner experiments were made in which 10 per cent cane sugar plus various other substances were added to solution A. For the experiment 500 cc of the solution were used. In determining the presence of tannase, 0.2 gram of the powdered mycelium was added to 75 cc of a 0.9 per cent tannic acid solution to which had been added 2 per cent toluene as an antiseptic. The results are given in table VIII.

²⁸ *Loc cit*

²⁹ H. Pottevin. La tannase. Diastase dédoublant l'acide gallotannique, *Compt rend de l'Acad des Sci*, cxvii, pp 1215-17, 1901

TABLE VIII
Aspergillus niger

COMPOSITION OF NUTRIENT SOLUTION	GALLIC ACID INCREASE
Solution B + 10 per cent sugar + 35 mgm ZnSO ₄ H ₂ O	None
Solution B + 10 per cent sugar + 20 mgm salicylic acid	None
Solution B + 10 per cent sugar + 1 gram gallic acid	None
Solution B + 10 per cent sugar + 200 mgm methyl salicylate	None
Solution B + 10 per cent sugar + 200 mgm ethyl salicylate	None
Solution B + 10 per cent sugar + 50 mgm methyl salicylate	None
Solution B + 10 per cent sugar + 50 mgm ethyl salicylate	None

Pottevin³⁰ states that tannase has the property of hydrolyzing methyl and ethyl salicylate. In my cultures the methyl or ethyl salicylate did not incite the development of the enzyme. The amount of salicylate present, however, was small. These concentrations may have been too weak to stimulate the production of the enzyme but probably the hydrolysis of these two substances is due to another enzyme. Salicylic acid also was used because of its relation to gallic acid, salicylic acid being a monohydroxybenzoic acid, while gallic acid is a trihydroxybenzoic acid. The former was necessarily used at a very low concentration, and was without effect. The zinc sulphate was used at a concentration which is stimulating to the fungus growth, as shown by Richards,³¹ but no formation of the tannase resulted.

Since in some of the experiments tannase was not produced when the organism was grown in a solution with the carbon supplied as cane sugar and gallic acid, an experiment was made with the carbon supplied only as gallic acid. At the same time experiments were made to determine the influence of certain glucosides. Solution B was used, and 500 cc of it placed in each of three liter flasks. These were plugged and sterilization made at 115° for ten minutes. To each of the three flasks was added gallic acid, amygdalin and salicin, respectively, and the culture solutions

³⁰ *Loc cit*

³¹ H. M. Richards. Die Beeinflussung des Wachstums einiger Pilze durch chemische Reize, *Jahrb f wiss Bot*, xxx, pp 665-88

then inoculated. The growth in gallic acid was rapid, but not heavy. In the solutions in which the glucosides were present, growth was at first very slow, but after the transformation of the glucosides had begun, growth was rapid. While it required eight days to develop the first felts in the two glucoside solutions, the second felts (after the removal of the first) developed in two days. The felts first formed were removed and treated according to the methods previously described, and the powdered mycelium then added to 100 cc. of a 0.90 per cent solution of tannic acid which contained also 2 per cent of toluene. After incubating two days at 40°C, the solutions were analyzed for gallic acid. The composition of the nutrient solutions and the gallic acid formed by the powdered mycelium are given in table IX.

TABLE IX
Aspergillus niger

COMPOSITION OF NUTRIENT SOLUTION	GALLIC ACID PRESENT	GALLIC ACID INCREASE
Solution B 500 cc. + 10 grams gallic acid	0.546	0.219
Solution B 500 cc. + 10 grams salicin	0.327	0
Solution B 500 cc. + 2 grams amygdalin	0.327	0

The control contained 0.327 gram gallic acid and 0.555 gram tannic acid.

In preceding tables it has been seen that gallic acid at certain concentration in the presence of 10 per cent sugar does not stimulate the formation of the enzyme tannase. When gallic acid is present alone as the source of carbon, the enzyme is produced. If tannic acid is a glucoside, it might be expected that the presence of another glucoside would stimulate the production of some tannase, but such was not the case. Nevertheless, glucosides were transformed by the organism. While the gallic acid stimulates the formation of the enzyme tannase, it does not do so as effectively as the tannic acid.

VI EFFECT OF CONCENTRATION OF GALLIC ACID ON PRODUCTION OF TANNASE

Since the amount of tannase produced is regulated by the relative concentrations of sugar and tannic acid it was deemed important to determine if the amount of the tannase produced could be in-

creased by increasing the concentration of the gallic acid. In the experiment, the results of which are given in table X, the usual methods were followed. Erlenmeyer flasks of 250 cc capacity were used and 100 cc of the nutrient solution. For determining the tannase activity of the various cultures 0.05 gram of the dried mycelium was added to 50 cc of 1 per cent tannic acid solution and the solutions incubated for forty-eight and eighty hours respectively at a temperature of 30°. The culture solutions were tested for their tannase content by precipitating any enzyme present with alcohol and collecting on a filter any precipitate. The test for the enzyme was made as before. Incubation was made at a temperature of 30° for ninety hours. The results in table X are the averages of two cultures.

TABLE X
Aspergillus niger

Effect of concentration of gallic acid on the production of tannase. Average period of incubation for mycelial powder 64 hours and for enzyme excreted 90 hours

COMPOSITION OF NUTRIENT SOLUTION	WEIGHT OF FUNGUS Av. OF 2 CULTURES	INCREASE IN GALLIC ACID		
		Mycelium Av. of 2	Enzyme excreted Av. of 2	Total increase in gallic acid
	gram	gram		gram
Solution B + 10 per cent sugar + 0.1 per cent gallic acid	0.240	0	0	0
Solution B + 10 per cent sugar + 0.5 per cent gallic acid	0.442	0.024	0	0.024
Solution B + 10 per cent sugar + 1.0 per cent gallic acid	0.324	0.045	0	0.045
Solution B + 10 per cent sugar + 1.5 per cent gallic acid	0.280	0.046	0	0.046

An examination of the above table reveals the fact that the enzyme tannase is produced when the concentration of gallic acid is 0.5 per cent in the presence of 10 per cent sugar. At a concentration of 1.0 per cent gallic acid in the presence of 10 per cent sugar the amount of tannase produced is double that produced when only 0.5 per cent gallic acid is present. In the presence of

10 per cent sugar the gallic acid, so far as my previous results³² show, is not at all absorbed from the nutrient solution. Why the increase in gallic acid should increase the amount of tannase produced is therefore difficult to explain. The writer is not prepared at present to offer an explanation.

VII SUMMARY

1 There is a progressive increase of tannase in the two organisms *Aspergillus niger* and *Penicillium* sp with increased concentration of tannic acid in Czapek's solution containing 10 per cent sugar.

2 In a full nutrient solution containing, as a source of carbon, 2 per cent tannic acid, the addition of cane sugar decreases the quantity of the tannase produced by the organism. The higher the concentration of sugar the lower the quantity of tannase produced.

3 *Aspergillus niger* produces more tannase or a more active tannase per unit weight than *Penicillium* sp.

4 The production of tannase in *Aspergillus niger*, *Penicillium* sp and *Penicillium rugulosum* is stimulated by tannic acid and gallic acid, only, and the former is more effective than the latter.

5 There is a progressive increase of tannase in *Aspergillus niger* with increased concentration of gallic acid in a nutrient solution containing 10 per cent sugar.

VIII DISCUSSION

Dox³³ in his excellent paper makes the following statement: "There is no evidence that enzymes not normally formed by the organism in demonstrable quantities can be developed by special methods in nutrition. The influence of adding a particular substance to the medium is, therefore, not to develop an entirely new enzyme, but to stimulate the production of an existing enzyme, which is normally formed under all conditions." In the light of Dox's results it seems somewhat surprising that the enzyme tannase should be formed only under special nutrition. It may be as Dox³⁴

³ *Loc cit*

³³ A. W. Dox, The Intracellular Enzymes of *Penicillium* and *Aspergillus*, *loc cit*

³⁴ A. W. Dox, Enzyme Studies of Lower Fungi, *Plant World*, xv, pp. 40-43, 1912

has stated concerning my work that the formation of the enzyme tannase is an exception to the general rule. The work of other investigators previously mentioned has indicated, however, that the production of certain enzymes in other organisms is governed entirely by the character of the nutrition.

Dox¹¹ has considered only the influence of external environment upon the formation of the enzymes. It is to be expected that protease, lipase, nuclease, inulase and perhaps some of the other enzymes would be produced because the substances which they transform are present in the mycelium. If the action of an enzyme is reversible and they are synthesizing agents, then the question arises "can the products of the decomposition induce the formation of enzyme?" In my experiments the only substance besides tannic acid capable of inducing the formation of the tannase is gallic acid, which is a decomposition product of tannic acid.

Might it be possible that all of the enzymes are produced only in response to the influence of the zymolyte or to the products of its decomposition present either in the nutrient solution or in the mycelium? There is a considerable amount of evidence indicating that one or the other is always present, but there is also evidence that certain enzymes are seemingly produced in the entire absence of the zymolyte or the products of its decomposition. The whole problem is a complex one and requires investigation.

¹¹ Enzyme Studies of Lower Fungi, *loc cit*

METHOD OF PREPARING ASH-FREE CASEIN AND PARACASEIN

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Casein or paracasein that is to be used in studying the relations of these compounds to mineral bases must be free from all such bases. The preparation of casein or paracasein really ash-free is much more difficult than has been commonly assumed. The so-called chemically pure casein furnished by chemical supply houses usually contains as much as 0.6 per cent of ash. The preparations used in various investigations in which the ash content has been reported rarely contain less than 0.2 per cent of ash and not infrequently more than 0.5 per cent.

The principal basic element in casein or paracasein preparations, as usually made, is calcium, and its presence is frequently due to the existence of a compound of calcium and casein containing 0.22 per cent Ca (equal to 0.31 per cent CaO) or, in case of paracasein, double this amount of Ca, as we shall show in another paper (this *Journal*, p. 223). These salts of casein and paracasein are insoluble in water but easily soluble in a 5 per cent solution of NaCl, while base-free casein and paracasein are insoluble both in water and in the brine solution.

When casein or paracasein is carefully precipitated by dilute acids from milk or from their lime-water solutions, the precipitate is apt to contain more or less of the above-mentioned caseinate or paracaseinate in addition to base-free protein. The precipitation of such calcium salts occurs most readily when the usual precautions in precipitating casein or paracasein from milk are most rigidly observed, that is, when excess of acid is avoided. We have examined casein preparations obtained from chemical supply houses and have found that some of them are soluble in 5 per cent solution of NaCl to the extent of 50 per cent or more of their weight.

PREPARATION OF ASH-FREE CASEIN

After trying different methods of preparing casein so as to contain a minimum amount of calcium, we have obtained the most satisfactory results by the method described below. We have been able to prepare casein containing only 0.06 per cent of ash, consisting largely of calcium phosphate, derived from the trace of calcium not removed and the phosphorus of the casein molecule. The amount of calcium present in 5 grams of such material was too small to determine quantitatively.

Our method of preparation is to dilute separator skim-milk with seven or eight times its volume of distilled water and carefully add dilute acetic acid (6 cc of glacial acetic acid diluted to 1 liter) until the casein separates completely, after which the clear solution is removed by siphon as soon as the precipitate settles. Distilled water is then added, the mixture stirred vigorously and the precipitate allowed to settle, after which the wash water is siphoned off. Water is again added and the casein is dissolved by adding, for each liter of milk used, 1 liter of dilute NH_4OH (6 cc of strong reagent diluted to 1 liter). When the solution is complete, the whole is filtered through a thick layer of absorbent cotton. The casein is then precipitated again with dilute acetic acid, the precipitate is allowed to settle, and is then washed, redissolved in dilute NH_4OH and filtered, the process of precipitation, washing, dissolving, etc., being repeated not less than four times. Finally an excess of strong NH_4OH (10 cc) is added and then, 20 cc of saturated solution of ammonium oxalate. The mixture is allowed to stand twelve hours or more. Calcium is precipitated as oxalate in very finely divided condition, too fine to permit its satisfactory removal by ordinary methods of filtration. Better aggregation of the precipitate can, however, be effected by means of centrifugal force. The centrifuged mixture is then filtered through double thickness of filter paper. The filtered solution is next treated with dilute HCl (10 cc HCl , sp gr 1.20, diluted to 1 liter) until the casein is precipitated. The precipitate is washed with distilled water until free from chloride and is then placed on a hardened filter paper in a Buchner funnel, as much water as possible being now removed by suction. The mass is next transferred to a large mortar and thoroughly triturated with 95 per cent alcohol. The

alcohol is then removed by suction on a Buchner funnel and the casein is then again placed in a mortar and triturated with absolute alcohol. Most of the alcohol is removed and the casein treated twice with ether in a mortar by trituration, the ether being removed each time by means of suction on a Buchner funnel. The material is then placed in a large evaporating dish and spread out in a layer as thin as possible, it is allowed to stand twelve hours or more in a warm place. The material is finally ground in a mortar until the particles pass a 40-mesh sieve and is dried for two days over H_2SO_4 in a desiccator under diminished pressure.

Three preparations made in this way were found to show an ash content of 0.10, 0.09 and 0.06 per cent, respectively. These preparations were insoluble in water and in 50 per cent alcohol, the first one was very slightly soluble in a 5 per cent solution of NaCl , but the two others were not.

When 1 gram of these casein preparations was treated with 10 cc of $\frac{N}{10}$ NH_4OH , NaOH or KOH and 90 cc of water, a clear solution was obtained, the casein dissolving completely. When to this solution a minute amount of a solution of a salt of barium, strontium or calcium was added, there developed promptly the opalescent appearance characteristic of casein solutions under such conditions.

Casein prepared in the manner described was analyzed with the following results: Moisture, 1.09 per cent. In the dry substance: Ash, 0.06, C, 53.50, H, 7.13, N, 15.80, P, 0.71, S, 0.72, O (by difference), 22.08 per cent.

PREPARATION OF ASH-FREE PARACASEIN

Separator skim milk was heated to 37°C and rennet extract (Hansen's) was added in the proportion of 0.12 cc per 1000 cc of milk. The mixture was allowed to stand until the precipitated paracaseinate had separated as completely as possible. The resulting curd was then stirred vigorously in order to break it into small pieces and hasten the separation of whey. When the curd had settled, the supernatant whey was removed by siphon. The paracaseinate was washed with distilled water several times and finally 5 liters of water added for each liter of milk originally used. Dilute NH_4OH (6 cc of strong reagent diluted to 1000 cc) was then added, as in the case of preparation of casein described above, and the mixture stirred until the paracaseinate was dissolved.

process of reprecipitating, washing and redissolving was continued as in the case of casein, the remaining calcium was finally separated by addition of ammonium oxalate and centrifuging as described above

One preparation made in this way contained 0.07 per cent of ash. One gram gave a clear solution when dissolved in 10 cc of $\frac{N}{10}$ NH_4OH and 90 cc of water.

One preparation, with a high ash content, gave the following results when analyzed: Moisture, 1.63 per cent. In the dry substance, ash, 0.61, C, 53.50, H, 7.26, N, 15.80, P, 0.83, S, 0.87, O (by difference), 21.13.

Another preparation with exceptionally low ash content gave the following results: Ash, 0.07, phosphorus, 0.71, sulphur, 0.72 per cent.

SUMMARY

Ash-free casein and paracasein are prepared by alternate precipitation with dilute acids and solution with dilute NH_4OH several times, the last portion of calcium being removed by precipitation with ammonium oxalate, after which the protein is precipitated with dilute acid and purified by treatment with water, alcohol and ether, being finally dried over H_2SO_4 under reduced pressure.

PREPARATION AND COMPOSITION OF BASIC CALCIUM CASEINATE AND PARACASEINATE

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The compound commonly known as basic calcium caseinate contains the largest amount of calcium in combination with casein. This is the compound that has been most frequently prepared and studied by investigators, beginning with Soldner¹. Varying results have been obtained by different workers, the percentage of Ca varying from 1.66 to 2.13 per cent (equal to 2.32 to 2.98 per cent CaO).

This compound can be prepared in two different ways: (1) By decomposing CaCO_3 with casein and (2) by treating casein with a solution of Ca(OH)_2 (lime-water).

Preparation of basic calcium caseinate by treating casein with CaCO_3

When casein is treated with CaCO_3 , the results of the reaction can be measured in two ways: (1) By weighing the CO_2 displaced and (2) by determining the amount of Ca in the resulting compound. Both methods were employed by us.

Ash-free casein prepared in the manner described in the previous article was placed in the flask of a Knorr CO_2 apparatus and an excess of CaCO_3 suspended in water was added. The CO_2 formed in the reaction was run into weighed bulbs containing KOH, and the increase of weight due to CO_2 determined at the end of the reaction. The results are given in Table I.

For the purpose of measuring the results of the reaction by determining the amount of Ca in the resulting compound, the casein was placed in a mortar and thoroughly triturated with an excess

¹ *Landw. Versuchsstat.*, xxxv, p. 351, 1888.

TABLE I

Amounts of CO₂ expelled from CaCO₃ by casein

AMOUNT OF DRY CASEIN USED	AMOUNT OF CO ₂ EXPELLED	CaO (AND Ca) FOR 100 GRAMS OF CASEIN EQUIVALENT TO CO ₂
grams	grams	grams
10	0 1900	2 42 (1 73 Ca)
10	0 1980	2 52 (1 80 Ca)
5	0 1054	2 68 (1 91 Ca)
5	0 1003	2 55 (1 81 Ca)
Average		2 54 (1 81 Ca)

of moist CaCO₃, the excess being removed by filtration at the end of the reaction. The filtrate was treated with 95 per cent alcohol, which was acid-free, until the calcium caseinate was precipitated, after which the precipitate was washed with alcohol and ether and dried at 120°C. A weighed portion of this compound was carefully ignited and the Ca in the resulting ash was determined, with the following results

TABLE II

Amount of Ca combining with casein when reacting with CaCO₃

WEIGHT OF CASEINATF	WEIGHT OF CaO	WEIGHT OF Ca	WEIGHT OF FREE CASEIN	CaO (AND Ca) FOR 100 GRAMS OF CASEIN
grams	grams	grams	grams	grams
0 4125	0 0102	0 0073	0 4052	2 52 (1 80 Ca)
0 5134	0 0124	0 0089	0 5045	2 46 (1 76 Ca)
0 3090	0 0077	0 0055	0 3035	2 54 (1 81 Ca)
0 4253	0 0104	0 0074	0 4179	2 49 (1 77 Ca)
Average 0 41505	0 010175	0 00726	0 4078	2 50 (1 78 Ca)

Preparation of basic calcium caseinate by treating casein with an excess of Ca(OH)₂

Weighed portions of casein were dissolved in an excess of Ca(OH)₂ solution. Phenolphthalein indicator was then added to the solution and HCl was run in until the solution became neutral. The solution was then dialyzed to remove the CaCl₂ formed in neutralization. The dialyzed solution was evaporated to dryness, the residue dried at 120°C and weighed. The determination of Ca was made, after ignition, with the following results

TABLE III

Amount of Ca combining with casein on treatment with $\text{Ca}(\text{OH})_2$

WEIGHT OF CASEINATE	WEIGHT OF CaO	WEIGHT OF Ca	WEIGHT OF FREE CASEIN	CaO (AND Ca) FOR 100 GRAMS OF CASEIN
grams	grams	grams	grams	grams
1 582	0 040	0 0286	1 5534	2 58 (1 84 Ca)
1 471	0 035	0 0250	1 4460	2 42 (1 73 Ca)
1 548	0 038	0 0271	1 5209	2 50 (1 78 Ca)
Average 1 534	0 0377	0 0269	1 5070	2 50 (1 78 Ca)

The three sets of figures presented in tables I, II and III indicate that casein combines with Ca to form a compound containing about 2.50 per cent of CaO (equal to 1.78 per cent of Ca), the compound in solution is neutral to phenolphthalein. Expressed in another form, 1 gram of casein combines with 9×10^{-4} gram equivalents of Ca.

Composition of basic calcium paracaseinate

Like casein, paracasein manifests its acid character by its power to liberate CO_2 from CaCO_3 , forming a calcium paracaseinate. The results of the reaction were measured by us, in the manner described above, in the case of casein. The average of many determinations indicates that paracasein unites with Ca to form a paracaseinate which is neutral to phenolphthalein and has the same general composition as the caseinate.

SUMMARY

Basic calcium caseinate and paracaseinate are prepared (a) by treating the ash-free protein with CaCO_3 and (b) by dissolving protein in $\text{Ca}(\text{OH})_2$ and neutralizing to phenolphthalein with HCl . In the first reaction, the amount of CO_2 displaced by the protein was determined and also the amount of Ca in the compound resulting. In the second reaction, the resulting compound was isolated, purified and its Ca content determined. The different sets of determinations agree in showing the compound to contain about 2.50 per cent of CaO (equal to 1.78 per cent of Ca) or 1 gram of protein combines with 9×10^{-4} gram equivalents of Ca.

PREPARATION AND COMPOSITION OF UNSATURATED OR ACID CASEINATES AND PARACASEINATES

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Compounds of casein with bases, in which less base is present than in basic calcium caseinate (this *Journal*, p 207), have been reported. Soldner¹ obtained a compound of casein and Ca containing 1.11 per cent of Ca (equal to 1.55 per cent of CaO), or, expressed in another form, 1 gram of casein combined with 5.55×10^{-4} gram equivalents of Ca. This compound is neutral to litmus but acid to phenolphthalein and has been commonly known as *neutral calcium caseinate*. This compound as prepared by Van Slyke and Hart² contains about 1.07 per cent of Ca (equal to about 1.50 per cent of CaO), or, 1 gram of casein combines with 5.35×10^{-4} gram equivalents of calcium. Courant³ believes that, in addition to the basic and neutral compounds of casein and Ca, a third exists, in which the Ca is present in about one-half the amount contained in the neutral compound and one-third that contained in the basic compound, he regards them as mono-, di-, and tri-calcium caseinates. Timpe⁴ reports a compound containing 0.961 per cent of Na (equal to 0.868 per cent of CaO or 0.62 per cent of Ca), or, 1 gram of casein combines with 3.1×10^{-4} gram equivalents of Ca. Long⁵ was able to dissolve 1 gram of casein in just one-half the amount of alkali required for the phenolphthalein neutralization and therefore inferred the existence of acid caseinates containing one-half the amount of base contained in

¹ *Landw. Versuchsstat.*, xxxv, p 351, 1888

² *Amer. Chem. Journ.*, xxviii, p 461, 1905

³ *Pflüger's Archiv f. d. ges. Physiol.*, p 109, 1891

⁴ *Arch. f. Hyg.*, xviii, p 1, 1893

⁵ *Journ. of Amer. Chem. Soc.*, xxviii, p 372, 1906

basic calcium caseinate The existence of such a combination is questioned by Robertson⁶

In the course of our work, we became convinced that casein forms compounds containing less base than any of those reported by other workers While we were at work on this point, an article by Robertson⁷ appeared in which was reported a combination of casein and NaOH, 1 cc of alkali combining with 0.877 gram of casein Our further work confirms Robertson's results, although we have used a different method of procedure In addition, we have been able to prepare and isolate several salts for analysis Our study of these individual salts shows that NH_4 , Na and K compounds possess properties very different from those of Ba, Ca and Sr We have prepared and studied two sets of compounds of casein with bases, in one of which 1 gram of casein combines approximately with 1.125×10^{-4} gram equivalents of base, while in the other 1 gram of casein combines with about 2.25×10^{-4} gram equivalents of base

We shall now take up the details of our experimental work in preparing unsaturated or acid caseinates of the bases of the more common alkalies and alkaline earths

The specific object in view was to ascertain the smallest quantity of base with which casein combines to form a definite salt In the volumetric work our method of procedure was as follows

In 200 cc of $\frac{N}{50}$ alkali, we dissolved 5 grams of pure casein as quickly as possible and then made the volume to 250 cc Each 50 cc of this solution therefore represented 1 gram of casein dissolved in 50 cc of $\frac{N}{50}$ alkali A preliminary or trial determination was next made in the following manner Into a 300 cc Erlenmeyer flask we measure 50 cc of the caseinate solution and then add, a drop at a time, some $\frac{N}{50}$ HCl, until we have used 5 cc, the contents of the flask being kept in constant agitation in order to prevent premature precipitation of casein After addition of 5 cc of acid a portion of the contents of the flask is centrifuged, in order to cause the sedimentation of precipitated casein, if any, a precipitate serving as an indicator A sedimentation tube of 50 cc capacity can be used, the precipitate collects in the lower V-shaped portion It is possible in this manner to detect the casein precipitated by 0.20 cc of $\frac{N}{50}$ HCl In case no casein is precipitated by the first addition of 5 cc of acid, another equal amount of acid is added and a portion of the mixture centrifuged, the process of adding

⁶ This Journal, 11, p 336, 1906

⁷ Journ of Physical Chem, xii, p 469 1909

5 cc portions of acid and centrifuging is continued until a permanent precipitate of casein is obtained. This shows, within 5 cc of $\frac{N}{50}$ HCl, how much acid is required to start definite precipitation of the casein. In order to ascertain the exact point more closely, another set of determinations is made, using 50 cc of the caseinate solution and adding in the same continuous manner an amount of $\frac{N}{50}$ HCl which is 5 cc less than the amount causing the first appearance of a permanent precipitate in the trial or preliminary determination. The acid is now added in small amounts with constant agitation of the liquid in order to prevent the premature separation of any precipitate, and centrifuged after the addition of each 0.25 cc. The first point at which a permanent precipitate appears is noted, the addition of acid is continued until all of the casein is precipitated and this point is also noted. In our work this method of determination was repeated several times with each combination of casein and alkali and three different casein preparations were used in preparing each caseinate.

We shall now present the results of our experimental work in connection with unsaturated or acid caseinates of, first, NH_4 , Na and K and, second, Ca, Sr and Ba.

Acid caseinates of NH_4 , Na and K

In the manner described above, we made numerous determinations in the case of preparations of 1 gram of base-free casein dissolved in 50 cc of the hydroxides of NH_4 , Na and K, respectively. In every case, irrespective of the alkali used, the volume of $\frac{N}{50}$ HCl required to cause the first sign of permanent precipitation was between 44.25 and 44.50 cc. In each case also the amount necessary to cause complete precipitation was 50 cc.

These results indicate that 1 gram of casein forms a soluble compound with NH_4 , Na and K, when combined with amounts of each somewhere between 1.10×10^{-4} and 1.15×10^{-4} gram equivalents, expressed as hydroxide, or, expressed in another form, 1 cc of $\frac{N}{10}$ alkali combines with an amount of casein somewhere between 0.87 and 0.91 gram. The proportion of basic element in each compound is approximately the following: NH_4 , 0.20 per cent, Na, 0.26 per cent, and K, 0.44 per cent. Caseinates combining with the amount of alkali base indicated contain the smallest known amount of base, according to our present knowledge. It seems proper, therefore, to suggest that such compounds be called *mono-basic caseinates*.

Preparation of mono-ammonium caseinate It seemed desirable that we should carry the work somewhat further and prepare one

pure compound, at least, in dry form for study. The NH_4 compound was chosen as the one offering least difficulty. The method of preparation was as follows:

In 2 liters of distilled water containing 250 cc of $\frac{N}{10}$ NH_4OH , 25 grams of base-free casein were dissolved. After solution was complete, we slowly added 125 cc of $\frac{N}{10}$ HCl , care being taken to agitate the mixture during the addition of the acid, in order to prevent premature precipitation of any casein. There was next added very cautiously $\frac{N}{50}$ HCl until a permanent precipitate began to appear, as shown by centrifuging the mixture at intervals. The solution was then filtered and measured. The amount of $\frac{N}{50}$ HCl required to precipitate the casein completely was determined in an aliquot part. Then one-third of this amount was added to insure the presence of only mono-basic caseinate. Any precipitate formed was removed by filtration and the filtrate was dialyzed until the NH_4Cl that had been formed in the reaction was completely removed. The resulting solution, containing mono-ammonium caseinate, was then precipitated by addition of acid-free alcohol. The precipitate was filtered, washed with acid-free alcohol and ether and dried at 120°C . In several preparations thus made, the amount of NH_4 was determined, the results are given in the following table:

TABLE I
Composition of mono-ammonium caseinate

AMOUNT OF CASEINATE USED	AMOUNT OF $\frac{N}{10}$ NH_4OH FOUND	RELATION OF CASEIN TO NH_4OH IN CASEINATE	PERCENTAGE OF NH_4 IN CASEINATE
grams	cc		
5.891	6.64	1 gm casein to 1.127×10^{-4} gm equivalents	0.203
4.870	5.38	1 gm casein to 1.105×10^{-4} gm equivalents	0.200
*4.000	4.30	1 gm casein to 1.075×10^{-4} gm equivalents	0.194
*3.000	3.16	1 gm casein to 1.053×10^{-4} gm equivalents	0.190

* Preparations of caseinates made by Mr. O. B. Winter

Acid caseinates of Ca, Sr and Ba

In making preparations of the caseinates of the alkali earth bases, difficulty was experienced for some time in obtaining concordant results. The trouble was finally found to be due to the presence of the chloride formed when the solution of the caseinate is treated with HCl . Such chlorides tend to cause precipitation

of the caseinates either by decreasing their solubility or, perhaps, by formation of double salts, consisting of the chloride in combination with the caseinate⁸ The difficulty of insolubility is readily overcome by removal of the chloride through simple dialysis before its amount is sufficient to cause precipitation To accomplish this we made use of the following process

In 200 cc of $\frac{N}{10}$ hydroxide of Ca, Sr or Ba, we dissolved 5 grams of casein and then diluted the solution to 250 cc A trial or preliminary determination was made by adding $\frac{N}{50}$ HCl to 50 cc of the caseinate solution in portions of 5 cc at a time, agitating constantly and, after each addition, testing for the presence of a precipitate by centrifuging a portion, until a precipitate appeared, just as in the case of preparing alkaline caseinates (p 212) Then to each of several flasks containing 50 cc of the caseinate solution, we added an amount of $\frac{N}{50}$ HCl that was 5 cc less than the amount causing the first appearance of a permanent precipitate in the preliminary trial The contents of the flask were then placed in dialyzing tubes, and, by frequent changes of the surrounding water, most of the soluble chloride that had been formed was removed The contents of one tube were used for another preliminary test An amount of acid less than that required to produce a precipitate in this second test was then added to all the tubes and the contents again dialyzed This operation was continued in the manner indicated in Table 216 (p 216)

In the manner described above, we have made numerous preparations of Ca, Sr and Ba caseinates, the averages of many results show that, in adding $\frac{N}{50}$ HCl to 50 cc of a caseinate solution containing 1 gram of casein dissolved in 50 cc of $\frac{N}{50}$ solution of hydroxide of Ca, Sr and Ba, 38.5 to 39.0 cc of $\frac{N}{50}$ HCl will be required to cause the first appearance of a permanent precipitate, also, the addition of only 44.5 cc will be required to cause the complete precipitation of the casein The remaining amount of base, equal to 5.5 cc of $\frac{N}{50}$ hydroxide, or 1.1 cc of $\frac{N}{10}$ hydroxide, appears to be held in combination in the insoluble compound

These results indicate the formation of two sets of compounds, when casein is dissolved in a hydroxide of Ca, Sr or Ba and this solution is neutralized with acid under the conditions of our experiments One set of compounds contains twice as much basic element as the other

Attention is called to additional details in the following statements

⁸ Pfeiffer and Modelski *Zeitschr f physiol Chem*, lxxxi, p 329, 19

1 In the di-basic compounds, as the results show, 1 gram of casein requires between 2.2×10^{-4} and 2.3×10^{-4} gram equivalents of hydroxide of Ca, Sr or Ba to form a compound which is soluble in water when there is not present any, or more than a trace of, soluble chloride of any of these elements. The addition

TABLE II

Illustration of method used in preparing acid caseinates of Ca, Sr and Ba

AMOUNT OF CASEIN IN SOLUTION	AMOUNT OF N 50 HYDROXIDE SOLUTION USED	AMOUNT OF N 50 HCL ADDED	SIGN OF FIRST PERMANENT PRECIPITATE	
gram	cc	cc		
1	50	30	precipitate	First trial
1	50	25	0	Dialyzed and used for next
1	50	30	0	Dialyzed and used for next
1	50	35	precipitate	
1	50	25	0	Dialyzed and used for next
1	50	30	0	Dialyzed and used for next
1	50	35	0	
1	50	40	precipitate	
1	50	25	0	Dialyzed and used for next
1	50	30	0	Dialyzed and used for next
1	50	35	0	Dialyzed and used for next
1	50	36	0	
1	50	37	0	
1	50	38	precipitate	
1	50	25	0	Dialyzed and used for next
1	50	30	0	Dialyzed and used for next
1	50	35	0	Dialyzed and used for next
1	50	37	0	Dialyzed and used for next
1	50	38	0	
1	50	39	precipitate	
1	50	25	0	Dialyzed and used for next
1	50	30	0	Dialyzed and used for next
1	50	35	0	Dialyzed and used for next
1	50	37	0	Dialyzed and used for next
1	50	38	0	Dialyzed and used for next
1	50	38.5	0	
1	50	39	precipitate	

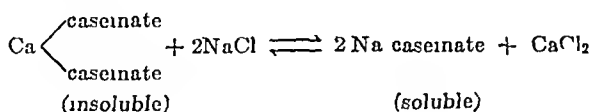
of even a small amount of a soluble salt of any of these elements to a solution of any of these di-basic caseinates causes the formation of a precipitate

2 In these di-basic compounds, 100 grams of casein combine (a) with 0.44 to 0.46 gram of Ca (equal to 0.62 to 0.64 gram of CaO), (b) with 0.96 to 1.01 grams of Sr (equal to 1.14 to 1.19 grams of SrO) or (c) with 1.51 to 1.58 grams of Ba (equal to 1.69 to 1.76 grams of BaO)

3 It is indicated that with the treatment described above 1 gram of casein combines with about 1.1×10^{-4} gram equivalents of the hydroxide of Ca, Sr or Ba to form an *insoluble* compound, when an acid is added in amount just sufficient to precipitate the casein completely. These compounds are regarded as *mono-basic*.

4 In these insoluble mono-basic compounds, 100 grams of casein combine approximately (a) with 0.22 gram of Ca (equal to 0.31 gram of CaO), (b) with 0.48 gram of Sr (equal to 0.57 gram of SrO) or (c) with 0.76 gram of Ba (equal to 0.85 gram of BaO)

5 These insoluble compounds possess some highly interesting properties. They are soluble in a 5 per cent solution of NH_4Cl , NaCl and KCl . This solubility is due to an exchange of bases, which, for the purpose, can be represented by the following reversible reaction



That the reaction is a reversible one is supported by the following experimental evidence. Mono-calcium caseinate was prepared and freed from soluble calcium salts by washing and dialysis. The compound was then dissolved in a 5 per cent solution of calcium-free NaCl . That an interchange of bases had taken place was shown by the fact, that when the brine solution of caseinate was dialyzed, calcium was found in the solution outside the dialyzing tube. This brine solution of caseinate was then dialyzed until free from calcium and was then filtered. A solution of CaCl_2 was then added to this dialyzed solution and at once a precipitate of calcium caseinate was produced. That this precipitate is a cal-

cium salt can be shown in two ways (1) By washing and dialyzing until free from soluble chloride and then igniting, calcium is found in the ash (2) By washing and dialyzing until free from soluble calcium, then redissolving in a 5 per cent solution of calcium-free NaCl and dialyzing, calcium is found to dialyze out of this brine solution of caseinate

Preparation of mono- and di-calcium caseinates In order to study the composition and properties of these compounds more fully, preparations of mono- and di-calcium caseinate were made. The following method was employed

In 800 cc of $\frac{N}{10}$ Ca(OH) there were dissolved 20 grams of base-free casein. To this solution were added 400 cc of $\frac{N}{10}$ HCl, the solution was then dialyzed to remove most of the resulting CaCl₂. Then $\frac{N}{10}$ HCl was added very cautiously under constant agitation of the mixture until a permanent precipitate began to appear, as shown by centrifuging. The solution was then dialyzed again and then more acid was added until a precipitate once more began to form. Alternate dialysis and addition of acid were continued until no more acid could be added without causing a precipitate. The amount of acid necessary to precipitate all of the casein was next determined in an aliquot portion, and one-third of this amount of acid was then added. The precipitated casein was filtered out and the filtrate was dialyzed. This solution contained di-calcium caseinate. The solution was divided, one portion being used for the preparation of di-calcium caseinate and the other for the mono-calcium caseinate.

In completing the preparation of the di-calcium caseinate, the salt was precipitated by addition of acid-free alcohol, the precipitate being washed in acid-free alcohol and ether, and then dried at 120°C. The composition of this preparation is given below in table IV.

In preparing the mono-calcium caseinate, the solution of di-calcium caseinate was treated with enough acid to precipitate three-fourths of the casein. The resulting precipitate was filtered, washed with water, acid-free alcohol and ether, and then dried at 120°C. The results in table III show the amount of Ca found in the preparation.

If we compare the results given in tables III and IV with the figures given in paragraphs (1), (2), (3) and (4) on page 217 it is obvious that the results embodied in these tables are lower. The higher results are obtained by the volumetric method and are believed to be nearer the truth, owing to the difficulty of preparing these caseinates in pure form. The values given by the volumetric method are the following: 1 gram of casein to 1.10 (to 1.15) $\times 10^{-4}$ gram equivalents of calcium for the mono-basic

TABLE III
Composition of mono-calcium caseinate preparation

AMOUNT OF COMPOUND USED	AMOUNT OF CaO (AND Ca) FOUND	PERCENTAGE OF CaO (AND Ca) IN COMPOUND	RELATION OF CASEIN TO CALCIUM IN COMPOUND
<i>grams</i>	<i>gram</i>		
5	0.0149 (0.0106Ca)	0.298 (0.213 Ca)	1 gm casein to 1.06×10^{-4} gm equivalents
5	0.0141 (0.0101Ca)	0.282 (0.201 Ca)	1 gm casein to 1.01×10^{-4} gm equivalents
5	0.0146 (0.0104Ca)	0.292 (0.209 Ca)	1 gm casein to 1.04×10^{-4} gm equivalents
Average	0.01453 (0.0104Ca)	0.291 (0.208 Ca)	1 gm casein to 1.04×10^{-4} gm equivalents

TABLE IV
Composition of di-calcium caseinate preparation

AMOUNT OF COMPOUND USED	AMOUNT OF CaO (AND Ca) FOUND	PERCENTAGE OF CaO (AND Ca) IN COMPOUND	RELATION OF CASEIN TO CALCIUM IN COMPOUND
<i>grams</i>	<i>gram</i>		
4.2825	0.0233 (0.0167Ca)	0.544 (0.39 Ca)	1 gm casein to 1.95×10^{-4} gm equivalents
4.1215	0.0235 (0.0168Ca)	0.572 (0.41 Ca)	1 gm of casein to 2.04×10^{-4} gm equivalents
Average 4.202	0.0234 (0.01675Ca)	0.558 (0.40 Ca)	1 gm of casein to 2.00×10^{-4} gm equivalents

caseinate, and 1 gram of casein to 2.2 (to 2.3) $\times 10^{-4}$ gram equivalents of calcium for the di-basic caseinate

PREPARATION AND COMPOSITION OF UNSATURATED OR ACID PARACASEINATES

In preparing acid paracaseinates of bases, the same volumetric method of procedure was followed as in case of the casein salts (p 215). The appearance of a precipitate in a centrifuged portion after addition of acid to an alkali solution of paracaseinate was made to serve as an indicator in regard to the end point of the reaction. We dissolved 5 grams of the ash-free paracaseinate in 200 cc of $\frac{N}{10}$ alkali, made up the solution to 250 cc and then brought to the end point by careful addition of $\frac{N}{10}$ HCl.

Acid paracaseinate of NH₄, Na and K

In the manner described, determinations were made in the case of base-free paracasein dissolved in hydroxides of NH₄, Na and K. In every case, irrespective of the alkali used, the volume of $\frac{N}{50}$ HCl required to cause the first sign of permanent precipitation when added to a solution of 1 gram of casein in 50 cc of $\frac{N}{50}$ alkali was between 38.5 and 39 cc, in each case, also, the amount required to cause complete precipitation was 50 cc.

These results show that 1 gram of paracasein combines with an amount of alkali somewhere between 2.2×10^{-4} and 2.3×10^{-4} gram equivalents, expressed as hydroxide, in forming soluble compounds with NH₄, Na and K, which are acid to both litmus and phenolphthalein. Expressed in another form, 1 cc of $\frac{N}{10}$ alkali, expressed as hydroxide, combines with an amount of paracasein somewhere between 0.435 and 0.455 gram. The proportion of basic element in each compound is approximately as follows: NH₄, 0.40 per cent, Na, 0.52 per cent, K, 0.88 per cent. The amount of each basic element in these paracaseinates is just double that present in the corresponding casein compounds.

The amount of acid required to precipitate completely the paracasein in these compounds is exactly equal to the alkali used to dissolve the paracasein, this fact indicates that there is not an additional paracaseinate, in insoluble form, containing less of these basic elements.

Preparation of mono-ammonium paracaseinate Mono-ammonium paracaseinate was isolated and prepared in dry form for further study in the manner already described in the preparation of mono-ammonium caseinate (p. 213). Care must be taken to use a paracasein preparation free from casein or salts of Ca, Sr, Ba, etc. A determination of the amount of NH₄ present in preparations thus made is given in Table V.

These results illustrate the difficulty of preparing the compounds pure, but indicate that the percentage of NH₄ is about double that found in the corresponding mono-ammonium caseinate.

Acid paracaseinates of Ca, Sr and Ba

In preparing paracasein salts of Ca, Sr and Ba, the presence of their chlorides causes much more trouble in respect to precipitation

TABLE V

Composition of mono-ammonium paracaseinate

AMOUNT OF PARACASEIN USED	AMOUNT OF $\frac{N}{N}$ NH_4OH FOUND	RELATION OF PARACASEIN TO NH_4OH IN PARACASEINATE	PERCENTAGE OF NH_4 IN PARACASEINATE
4	8.20	1 gm paracasein to 2.05×10^{-4} gm equivalents	0.37
4	7.98	1 gm of paracasein to 2.00×10^{-4} gm equivalents	0.36

than in case of the caseinates. Special care must be taken to prevent the accumulation of chlorides of these elements. By sufficiently frequent dialysis, it was possible to obtain the results reported below. Another point in connection with paracasein is the fact of its slow rate of solution in the hydroxides of Ca, Sr and Ba, on this account we used 400 cc of $\frac{N}{N}$ hydroxide to dissolve 5 grams of paracasein, making the volume up to 500 cc with water.

Trial or preliminary determinations were made in the same manner as with casein (p 215), in order to determine the amount of $\frac{N}{N}$ HCl required to precipitate the paracasein in the absence of CaCl_2 , SrCl_2 or BaCl_2 . The specific details employed and results obtained are indicated in Table VI (p 222).

The results obtained by the method of procedure indicated above showed that the amount of $\frac{N}{N}$ HCl which must be added to a solution of 1 gram of paracasein in 100 cc of $\frac{N}{N}$ hydroxide of Ca, Sr or Ba in order to cause the first sign of permanent precipitation was between 77.25 and 77.5 cc to completely precipitate all of the paracasein, 88.5 cc were required. The figures are the same irrespective of the hydroxide used.

These results indicate the formation of two sets of compounds when paracasein is dissolved in a $\text{Ca}(\text{OH})_2$, $\text{Sr}(\text{OH})_2$ or $\text{Ba}(\text{OH})_2$ and this solution is neutralized with acid under the conditions of our experiments. One set of compounds contains twice as much base as the other, corresponding to the two sets of casein compounds. The following statements call attention to additional details.

1. In the di-basic compounds, the results show that 1 gram of paracasein requires between 4.5×10^{-4} and 4.55×10^{-4} gram

TABLE VI

Illustration of method of preparing acid paracaseinates of Ca, Sr and Ba

AMOUNT OF PARACASEIN IN SOLUTION	AMOUNT OF N 56 HYDROXIDE SOLUTION USED	AMOUNT OF N 56 HCL ADDED	PRECIPITATION	
gram	cc	cc		
1	100	80 0	precipitate	First trial
1	100	75 0	0	Dialyzed and used for next
1	100	80 0	precipitate	
1	100	75 0	0	Dialyzed and used for next
1	100	76 0	0	Dialyzed and used for next
1	100	77 0	precipitate	
1	100	75 0	0	Dialyzed and used for next
1	100	76 0	0	Dialyzed and used for next
1	100	76 5	0	Dialyzed and used for next
1	100	77 0	precipitate	
1	100	75 0	0	Dialyzed and used for next
1	100	76 0	0	Dialyzed and used for next
1	100	76 5	0	Dialyzed and used for next
1	100	77 0	0	Dialyzed and used for next
1	100	77 5	precipitate	
1	100	75 0	0	Dialyzed and used for next
1	100	76 0	0	Dialyzed and used for next
1	100	76 5	0	Dialyzed and used for next
1	100	77 0	0	Dialyzed and used for next
1	100	77 25	0	Dialyzed and used for next
1	100	77 50	precipitate	

equivalents of hydroxide of Ca, Sr or Ba to form a compound which is soluble in pure water. These compounds are easily precipitated from their water solutions by a minute amount of a soluble salt of Ca, Sr or Ba.

2 In these di-basic compounds, 100 grams of paracasein combine approximately (a) with 0.90 gram of Ca (equal to 1.26 grams of CaO), (b) with 1.97 grams of Sr (equal to 2.33 grams of SrO) or (c) with 3.09 grams of Ba (equal to 3.45 grams of BaO).

3 It is indicated that, with the treatment described above,

1 gram of paracasein combines with about 2.3×10^{-4} gram equivalents of the hydroxide of Ca, Sr or Ba to form an *insoluble* compound. These compounds are regarded as mono-basic paracaseinates.

4 In these insoluble mono-basic paracaseinates, 100 grams of paracasein combine approximately (a) with 0.46 gram of Ca (equal to 0.64 gram of CaO), (b) with 1.01 grams of Sr (equal to 1.19 grams of SrO) or (c) with 1.58 grams of Ba (equal to 1.76 grams of BaO).

5 Mono-basic paracaseinates of Ca, Sr and Ba are completely soluble in warm 5 per cent solution of NH_4Cl , NaCl and KCl . This solubility is due to interchange of bases, just as in the case of caseinates (p 217), the reaction was studied experimentally with paracaseinates and the same results obtained as in the case of the caseinates.

6 A comparison of the composition of the caseinates and paracaseinates shows that twice as much base is present in paracaseinates as in the corresponding caseinates. This is easily seen in the following table.

TABLE VII

Comparison of composition of caseinates and paracaseinates
Amount of basic element combined with 100 grams of casein or paracasein

BASIC ELEMENT	IN MONO-BASIC CASEINATE	IN MONO BASIC PARACASEINATE	IN DI-BASIC CASEINATE	IN DI BASIC PARACASEINATE
Ca	0.22	0.46	0.44 to 0.46	0.90
Sr	0.48	1.01	0.96 to 1.01	1.97
Ba	0.76	1.58	1.51 to 1.58	3.09

Preparation of mono- and di-calcium paracaseinates In order to study the composition and properties of these compounds further, preparations of the mono- and di-calcium paracaseinates were made. The first steps in making these compounds are the same. An excess of ash-free paracasein is agitated with lime-water until a saturated solution is formed, the undissolved paracasein being removed by filtration. To the solution, $\frac{N}{20}$ HCl is added until a permanent precipitate begins to appear. The solution is again filtered and then dialyzed. Alternate addition of acid and dialysis are continued until no more acid can be added after dialysis without causing precipitation. The amount of $\frac{N}{20}$ HCl required to

precipitate all the paracasein is next determined in an aliquot portion, and one-third that amount of acid is added. The solution is then filtered and dialyzed. This solution contains di-calcium paracaseinate. This solution is divided into two portions, in one the di-calcium paracaseinate is precipitated by addition of acid-free alcohol, the precipitate being washed with acid-free alcohol and ether and being dried at 120°C . This preparation was found to contain between 4.2×10^{-4} and 4.6×10^{-4} gram equivalents of Ca for 1 gram of paracasein.

In the second portion of di-calcium paracaseinate solution enough $\frac{N}{5}$ HCl is very slowly added to precipitate three-fourths of the paracasein in solution. The precipitate is mono-calcium paracaseinate, this is filtered, washed with acid-free alcohol and ether and dried at 120°C . Before being washed with alcohol, the precipitate is completely soluble in 5 per cent solution of NaCl. This compound, mono-calcium paracaseinate, is identical in its properties with the brine-soluble compound formed in Cheddar cheese, to which attention was first called by Van Slyke and Hart under the expression, "salt-soluble compound." Attention will be more fully called to this compound in another paper (this *Journal*, p 231). An analysis of this preparation showed it to contain between 2×10^{-4} and 2.3×10^{-4} gram equivalents of Ca for 1 gram of paracasein.

SUMMARY

1 Acid or unsaturated caseinates and paracaseinates of NH_4 , Na and K are prepared by dissolving the ash-free protein in $\frac{N}{5}$ hydroxide and neutralizing very carefully by successive additions with $\frac{N}{5}$ HCl, the end point being obtained by centrifuging a portion of the mixture in order to cause sedimentation of any precipitated protein, the desired end point is the first sign of a permanent precipitate. Protein precipitated by 0.20 cc of $\frac{N}{5}$ HCl can thus be detected.

2 Results show that 1 gram of casein forms a soluble compound with NH_4 , Na or K, corresponding to amounts of hydroxide between 1.10×10^{-4} and 1.15×10^{-4} gram equivalents, or 1 cc of $\frac{N}{5}$ alkali, expressed as hydroxide, combines with 0.87 to 0.91 gram of casein. Corresponding paracaseinates are formed but they contain twice the amount of basic element present in the caseinates.

Such compounds are called mono-basic Mono-ammonium caseinates were prepared in dry form and studied

3 Acid or unsaturated caseinates and paracaseinates of Ca, Sr and Ba are prepared by dissolving ash-free casein in $\frac{N}{5}$ hydroxide and proceeding as in case of alkali compounds, except that when the first sign of a permanent precipitate appears on the addition of HCl, the mixture is dialyzed to remove the chloride formed, because such chloride precipitates the caseinate or paracaseinate

4 Two sets of compounds are formed with Ca, Sr and Ba, mono- and di-basic In the di-basic caseinates, which are soluble, 1 gram of casein combines with 2.25 gram equivalents expressed as hydroxide These compounds are easily precipitated by soluble salts of Ca, Sr or Ba In the mono-basic caseinates, which are insoluble, 1 gram of casein combines with 1.125×10^{-4} gram equivalents expressed as hydroxide In the paracaseinates, twice the amount of base combines with the protein molecule, 1 gram of paracasein combines with 4.50 gram equivalents expressed as hydroxide in the di-basic compounds and with 2.35 in the mono-basic

5 Mono-basic caseinates and paracaseinates are insoluble in water but soluble in warm 5 per cent solution of NaCl, NH_4Cl , KCl, etc The solubility is due to an exchange of bases, the reaction, for example, between mono-calcium caseinate and NaCl results in the formation of the soluble sodium caseinate and CaCl_2 . The reaction is reversible

VALENCY OF MOLECULES AND MOLECULAR WEIGHTS OF CASEIN AND PARACASEIN

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In the case of the compound of casein and Ca which is neutral to phenolphthalein, it is found that 1 gram of casein combines with 9×10^{-4} gram equivalents of Ca. In the case of the mono-ammonium caseinate, the combination is in the proportion of 1 gram of casein to a value between 1.1×10^{-4} and 1.15×10^{-4} gram equivalents expressed as hydroxide (this *Journal*, page 213). Now, since we have one compound of known composition and another of approximately known composition, it should be possible by applying the rule of constant proportions to determine the true composition of the mono-basic caseinate and also the number of valencies satisfied in the caseinate that is neutral to phenolphthalein.

We have reason to believe that the proportion, 1 gram of casein to 1.125×10^{-4} gram equivalents of alkali hydroxide, is the true value, since, first, this lies between the two limits (1.10 and 1.15) found in our volumetric work, and, second, this figure agrees with that found by assuming a valency of 8 for the basic calcium caseinate, in which 1 gram of casein combines with 9×10^{-4} gram equivalents of Ca. Thus, if the valencies satisfied are 8, the proportion becomes 1 gram of casein to 1.125×10^{-4} gram equivalents of alkali, expressed as hydroxide, for monobasic caseinates. If, however, we were to assume that the number of valencies in the basic compound is 7 rather than 8, then the mono-basic salt would, theoretically, have the composition, 1 gram of casein to 1.285×10^{-4} gram equivalents of alkali, expressed as hydroxide, a value too high for our analytical results. If, on the other hand, we were to assume the numbers of valencies in the basic compound to be 9

(instead of 8), then the proportion in the mono-basic compound would become 1 gram of casein to 1×10^{-4} gram equivalents of alkali, expressed as hydroxide, a value too low for our analytical results obtained with mono-ammonium and other alkali caseinates. Therefore, assuming 8 as the true valency of basic calcium caseinate gives the value, 1 gram of casein to 1.125×10^{-4} gram equivalents of alkali, expressed as hydroxide, a result which agrees with the volumetric results obtained in the case of the mono-alkali caseinates.

Using the sulphur content as a basis on which to calculate the molecular weight of casein, we have $n \left(\frac{32.07}{0.72} \right) 100 = n4454 +$

If the value of n is 2, the molecular weight becomes 8908, which is in close agreement with the value previously found, 8888.

Using the amount of phosphorus in casein as a basis for calculating the molecular weight, we have $n \left(\frac{31.04}{0.71} \right) 100 = n4372 -$, which becomes 8744 if the value of n is 2.

On the basis of 8 representing the true number of valencies satisfied in the basic calcium caseinate molecule, the molecular weight of casein is $\left(\frac{1}{1.125 \times 10^{-4}} \right)$ or 8888 +. Robertson reaches similar results¹ by deducing the molecular weight of casein in several different ways. This would also make the equivalent weight of casein equal to $\frac{8888}{8}$ or 1111. This value is in close agreement with the equivalent weight assigned by other workers to casein prepared from cow's milk. Laqueur and Sackur give about 1135,² Matthaios-poulos gives 1131.5,³ Long gives 1124.⁴

As a result of the work done by us it would seem possible, theoretically, to prepare a series of not less than eight combinations of casein with each of the basic elements studied. According to what we have reason to believe at the present time, not less than four of these combinations have been prepared. Using the calcium compounds for illustration we have the following series

¹ *Journ. of Physical Chem.*, xv, p. 179, 1911

² *Hofmeister's Beiträge*, iii, p. 193, 1902

³ *Zeitschr. f. anal. Chem.*, xlvii, p. 492, 1908

⁴ *Journ. Amer. Chem. Soc.*, xxviii, p. 372, 1906

NAME OF COMPOUND	GRAMS OF Ca FOR 100 GRAMS OF CASEIN	VALENCIES SATISFIED
Mono-calcium caseinate	0.22 (equal to 0.31 CaO)	1
Di-calcium caseinate	0.44 (equal to 0.62 CaO)	2
Neutral calcium caseinate	1.07 (equal to 1.50 CaO)	5
Basic calcium caseinate	1.78 (equal to 2.50 CaO)	8

It is noticeable that, in this series, compounds are absent representing valencies of 3, 4, 6 and 7. Whether such compounds can be prepared we cannot say at present.

VALENCY OF PARACASEIN MOLECULE AND MOLECULAR WEIGHT OF PARACASEIN

In the case of basic calcium paracaseinate, the compound that is neutral to phenolphthalein, it is found that 1 gram combines with 9×10^{-4} gram equivalents of Ca, while in the case of mono-ammonium paracaseinate, the combination is in the ratio of 1 gram of paracasein to a value between 2.2×10^{-4} and 2.3×10^{-4} gram equivalents (this *Journal* p. 223). According to the rule of constant proportions, the number of valencies satisfied in the first compound would be between $\frac{9}{2.2}$ and $\frac{9}{2.3}$ or 4. The molecular weight of paracasein would, therefore, be $\left(\frac{1}{2.25 \times 10^{-4}}\right)$ or 4444+. Our results indicate that the molecular weight of casein, 8888, is just twice that of paracasein, 4444.

Calculated on the basis of the sulphur content, the molecular weight of paracasein would be $n \left(\frac{32.07}{0.72}\right) 100 = n4454+$, on the basis of the phosphorus content, we should have $n \left(\frac{31.04}{0.71}\right) 100 = n4372-$. The value of n would seem to be 1 and each molecule of paracasein would contain one atom each of sulphur and phosphorus.

Theoretically, it should be possible to make a series of four salts of paracasein. We have prepared three—those in which 1, 2 and 4 valencies are satisfied (pp. 219, 223).

In connection with the relative molecular weights of casein and paracasein, the facts indicate that the action of the principal

enzyme contained in rennet-extract splits the casein molecule into two molecules of paracasein, an effect opposite that advocated by some who believe that the paracasein molecule is a larger aggregation than that of casein. The following experiment supports our view.

Five grams of casein are dissolved in 250 cc of $\frac{N}{50}$ KOH. Using the volumetric method given elsewhere (this *Journal*, p. 212), it is found that 44.5 cc of $\frac{N}{50}$ HCl could be added to 50 cc of the caseinate solution, containing 1 gram of casein, before a permanent precipitate begins to appear. To another 50 cc of caseinate solution a few drops of neutral rennet-extract are added. Under the conditions of the experiment, no precipitate or curd is produced by the action of the rennet-enzyme. After a few minutes, some $\frac{N}{50}$ HCl is added and it is found that a permanent precipitate begins to form as soon as we add only 39 cc of $\frac{N}{50}$ HCl.

We have in hand a more extended investigation relating to the action of rennet-enzyme upon casein, the results of which will be published later.

SUMMARY

On the basis of the analytical results obtained in the study of the composition of the mono-basic and basic caseinates and paracaseinates, the molecular weight of casein is 8888, that of paracasein, 4444. The valency of the protein molecule in basic caseinates is 8, in basic paracaseinates, 4.

COMPOSITION AND PROPERTIES OF THE BRINE-SOLUBLE COMPOUND IN CHEESE

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(Received for publication, February 4, 1913)

During the manufacture and ripening of Cheddar cheese and of many other kinds of cheese, there is always found a protein that is soluble in a warm 5 per cent solution of NaCl. The existence of such a substance in Cheddar cheese was first brought to attention by work done in this laboratory.¹ The presence of this brine-soluble protein was shown to be associated in some way with the formation of acid in the cheese and, on the basis of some early experiments, VanSlyke and Hart were led to conclude erroneously that the substance consists of a combination of paracasein and lactic acid (called by them paracasein mono-lactate), which by the addition of more lactic acid becomes insoluble in dilute brine solution, forming a compound which they mistakenly regarded as paracasein di-lactate. As a result of later work,² they changed their first views and came to the conclusion that the so-called paracasein mono-lactate is simply the uncombined protein, paracasein, and that the so-called paracasein di-lactate is a compound of paracasein and lactic acid (1 gram of paracasein uniting supposedly with about 0.5 cc of $\frac{N}{10}$ acid). It may be stated here, in passing, that it was later shown by L. L. VanSlyke and D. D. VanSlyke³ that the protein casein does not unite with acids to form insoluble compounds but that the action is simply one of *adsorption*, by which more or less acid is taken from the surrounding solution and concentrated upon the surface of the solid particles of protein, in other words, it was shown that casein or paracasein mono-lac-

¹ *Amer Chem Journ*, xxviii, p 411, 1902

² *Ibid*, xxxiii, p 461, 1905

³ *Ibid*, xxxviii, p 383, 1907

tate and di-lactate have no existence as applied to the compound in question. It still remained, therefore, to find out what the brine-soluble substance really is, and work was continued along this line by the writers ⁴. We noticed that calcium is always to be found associated with the brine-soluble substance when it is separated from the other cheese constituents by extraction with a solution of calcium-free NaCl after previous removal of all water-soluble constituents. This fact suggested the possibility that the brine-soluble substance might be a combination of paracasein and calcium, containing less calcium than had been previously found in any combination of this element with paracasein. On the basis of such a possibility, it could be explained that with the formation of increased amounts of lactic acid in cheese-making, as a result of the bacterial decomposition of milk sugar, the acid would combine with more or less of the calcium contained in calcium paracaseinate, resulting in the production of a paracaseinate containing less calcium. This suggestion was strengthened by the fact that in Camembert cheese, the brine-soluble compound is formed during certain stages of the manufacturing process but soon disappears, its formation and disappearance being explained as follows, according to Bosworth ⁵. The brine-soluble substance is at first formed in Camembert cheese, as also in the case of Cheddar cheese, but, owing to the method of making this type of cheese, more acid is allowed to form in Camembert cheese, and, as a consequence, the brine-soluble substance loses its calcium and becomes free paracasein, which is insoluble in brine solution. Therefore, in the manufacture of Camembert cheese, it is found that after the first few hours the cheese contains no brine-soluble material, and, what is also significant, all the calcium is found in the water extract. The relation between the brine-soluble substance and the calcium found in the brine extract in the two types of cheese is illustrated in Table 1.

The question necessarily suggests itself whether the calcium always found in the brine-soluble extract of cheese is not there incidentally in a mechanical state rather than in a combination with paracasein. In order to study this question, the following work was done.

⁴ Technical Bulletin No. 4, 1907, New York State Agric. Exp. Sta.

⁵ Technical Bulletin No. 5, 1907, New York State Agric. Exp. Sta.

Twenty-five grams of cheese were ground with sand and extracted with water at about 55°C, using 150 cc portions until the extract amounted to 1000 cc. The residue, containing the brine-soluble substance, was placed in a dialyzing apparatus and allowed to dialyze to insure the removal of all soluble calcium. Sodium chloride was then added to the contents of the dialyzing tube, which was then placed in a beaker of water and allowed to remain four hours. Upon adding ammonium oxalate to some of the water in the beaker, a precipitate of calcium oxalate appeared. This result leads to the belief that the Ca is present in combination in an insoluble form and that an interchange takes place between it and Na, when the insoluble compound is treated with NaCl solution.

TABLE I
Comparison of changes in Cheddar and Camembert cheese

AGE OF CHEESE	KIND OF CHEESE	TOTAL NITROGEN IN THE FORM OF BRINE-SOLUBLE COMPOUND	PER CENT OF TOTAL CALCIUM FOUND IN BRINE-SOLUBLE COMPOUND
		per cent	
When curd was cut	Cheddar	3 13	trace
When curd was cut	Camembert	6 72	trace
Ten hours	Cheddar	96 00	27 96
Ten hours	Camembert	94 00	17 76
Two days	Cheddar	68 87	24 47
Two days	Camembert	4 39	trace
Four months	Cheddar	43 09	24 28

In order to throw further light on the character of the brine-soluble compound, a study was made of the solvent effect of several different chlorides. One kilogram of Cheddar cheese was ground fine, thoroughly mixed, and then 25-gram portions were ground with sand, placed in bottles and extracted with water in the manner described in the preceding paragraph. The residues were then extracted with solutions of chlorides and the results given in the following table were obtained. The solutions of the salts were used in such strengths that 1000 cc contained equivalent gram molecules. In the case of the weakest solution, extraction was continued as long as appreciable amounts of protein were obtained in the extract, 4000 cc being used, the results in these cases are given for each 1000 cc of extract as well as for the total.

TABLE II

Solvent effect of neutral chlorides on the brine-soluble compound in cheese

STRENGTH OF SOLUTION GRAM EQUIVALENTS PER 1000 cc	AMOUNT OF EXTRACT cc	PERCENTAGE OF TOTAL N IN WATER INSOLUBLE RESIDUE OF CHEESE EXTRACTED BY					
		NaCl	NH ₄ Cl	KCl	MgCl ₂	BaCl ₂	CaCl ₂
1 0	1000	68 57	67 62	50 47	63 81	0	0
0 8	1000	69 29	65 24	50 47	48 33	0	0
0 6	1000	56 19	56 43	45 95	lost	0	0
0 4	1000	51 43	51 19	44 52	23 57	0	0
0 2	1st 1000	47 62	49 05	40 95	4 00	0	0
0 2	2nd 1000	13 33	10 48	13 90	5 24	0	0
0 2	3rd 1000	2 95	4 10	2 00	1 29		
0 2	4th 1000	trace	trace	trace			
Total	4000	63 90	63 66	56 85			

In connection with the data in the preceding table, attention is called to certain phases of the results

1 The chlorides of Ba and Ca have no solvent effect. The chloride of Mg in strong molecular concentrations acts much like the chlorides of the alkalis, while in lower molecular concentrations its solvent power is greatly reduced.

2 Sammis and Hart⁶ attempted to study the solvent effect of these salts on the same material, but reached results not concordant with one another and not in agreement with ours. While we used solutions of such strength as to show the relation existing between the solvent action of the salt solution and its molecular concentration, they used solutions containing a uniform percentage by weight of different salts and extracted in every case with the same volume of solution. By using solutions of different salts having the same percentage composition by weight, but with a different molecular concentration, one would, under the circumstances, expect to obtain only discordant results, because the solvent effect of the solution is apparently a result of the mass action of the salt in solution, as shown by us (this *Journal*, p 217). If Sammis and Hart had in their work continued extraction until no more solvent effect was appreciable, their results would have been in satisfactory agreement with ours. This is strikingly

⁶This *Journal*, vi, p 181, 1909

shown in the above table in the case of the 0.2 N solutions, by continued extraction the total amounts extracted are found to be essentially the same as in the more concentrated solutions

Identity of the brine-soluble compound of cheese with mono-calcium paracaseinate

We have shown (this *Journal*, p 223) that paracasein combines with Ca to form a compound insoluble in water but soluble in 5 per cent solution of NaCl (Na replacing Ca). In this compound we have shown that 1 gram of paracasein is in combination with 2.25×10^{-4} gram equivalents of Ca. Indications pointed to the identity of the brine-soluble substance of cheese with this mono-calcium paracaseinate, and it remained to ascertain whether the protein part of the molecule in these two compounds is the same. In order to accomplish this, a preparation of the protein in the brine-soluble compound was made from cheese, and its composition and properties were studied.

One kilogram of Cheddar cheese was ground fine and then extracted with numerous portions of distilled water at about 55°C in order to remove all soluble compounds. The residue was then extracted with many portions of a 5 per cent solution of NaCl and filtered, first through absorbent cotton and then through paper. Dilute acetic acid was then added, giving a heavy precipitate, which was washed with water, redissolved in dilute ammonia and again precipitated with acid. The process was then completed as in the preparation of casein (this *Journal*, p 204). The preparation on analysis gave the following results: Moisture, 2.32, ash, 0.25 per cent. In the dry substance, C, 52.97, H, 7.15, N, 15.82, P, 0.75, S, 0.78, O (by difference), 22.28.

A study of the properties of this substance gave the following results:

1. The substance is found to act as an acid in combining with bases.

2. It decomposes CaCO_3 and gives a compound in which 100 grams of substance combine with the equivalent of 2.52 grams of CaO (equal to 1.80 grams of Ca), or, 1 gram of substance combines with 9×10^{-4} gram equivalents of Ca.

3 The solution of this calcium compound is neutral to phenolphthalein

4 Measured by the volumetric method it was found to form a compound with ammonium represented by the combination of 1 gram of substance with 2.3×10^{-4} gram equivalents, expressed as hydroxide

5 With Ca it forms a compound, soluble in 5 per cent solution of NaCl but insoluble in water, which contains 1 gram of substance combined with 2.3×10^{-4} gram equivalents of Ca

6 It forms also a compound with Ca that is soluble in water, containing 1 gram of substance combined with 4.5×10^{-4} gram equivalents of Ca

In view of the marked agreement of the composition and properties of the brine-soluble substance, formed in cheese, with the compound, mono-calcium paracaseinate, as prepared by us, there is good reason to believe that the brine-soluble substance is mono-calcium paracaseinate, having the composition of 1 gram of paracasein combined with 2.25×10^{-4} equivalents of Ca

SUMMARY

In many kinds of cheese there is always present a protein soluble in warm 5 per cent solution of NaCl. Previous efforts to determine the exact relation of this substance to casein or paracasein have resulted in erroneous conclusions. An extended study of its properties and composition indicates the substance to be mono-calcium paracaseinate, formed from calcium paracaseinate by removal of part of its Ca through lactic acid produced in the process of cheese making as a result of the action of lactic acid bacteria upon the milk sugar.

ON THE RATE OF EXTRACTION OF A PROTEIN (SAL-MINE) FROM DESICCATED TISSUE BY AN AQUEOUS SOLVENT

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(Received for publication, February 5, 1913)

The testicles of the Pacific salmon (*Oncorhynchus tshawytscha*, Wahlbaum) which had been preserved and hardened in 50 per cent alcohol for two years preceding the experiment, were minced and the spermatozoa shaken out into a large bulk of distilled water. The suspension of sperm which was thus obtained was decanted from the bulk of the connective tissue and then filtered through glass wool. The spermatozoa were then agglutinated by the addition of 80 cc. of $\frac{M}{3}$ acetic acid per liter of suspension and allowed to settle out, the supernatant fluid being removed by decantation. The spermatozoa were then suspended in a volume of 95 per cent alcohol equal to the volume of the original suspension. After settling, the supernatant fluid was syphoned off and replaced by the same volume of 95 per cent alcohol. After again allowing the sperm to settle and removing the supernatant fluid, they were suspended in a volume of ether equal to one-half of the volume of the original aqueous suspension. After again allowing the sperm to settle, the supernatant ether was removed by decantation, the sperm collected in a cloth and the greater part of the ether squeezed out of them. They were then spread out upon bibulous paper to dry in the air of a warm room. The desiccated spermatozoa were thus obtained in the form of a yellowish powder containing some coarse particles. The powder was sifted through a very fine sieve, and the portion which passed through the sieve was employed in the experiment which is about to be described.

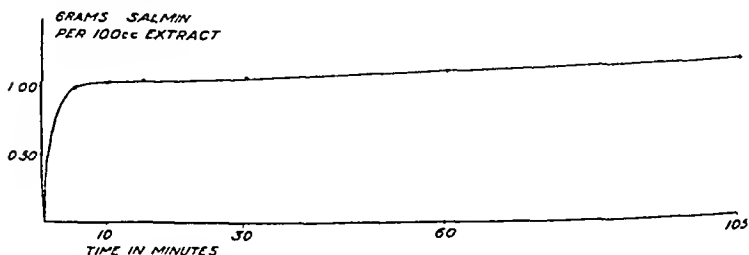
One hundred and fifty cubic centimeters of an aqueous solution of hydrochloric acid containing 1.35 per cent of HCl were placed

in a beaker, of which the diameter of the base was 7.5 cm, and agitated by a glass rod which was bent to an angle of 45° from the vertical and the tip of which, when rotated, described a circle of about 7 cm diameter. The rod was rotated by a small motor at the very nearly constant rate of 1600 revolutions per minute. The temperature was that of the room.

Forty-five grams of the dried sperm were dropped into this fluid while stirring, and 150 cc more of the 1.35 per cent HCl were immediately added. The entire process of dropping in the sperm and adding more fluid occupied less than fifteen seconds.

At the intervals stated below samples of the mixture were almost instantaneously withdrawn by means of a 20 cc pipette provided with a rubber bulb. The samples were then very rapidly filtered under pressure through small dry pads of asbestos in Gooch crucibles, the filtrates being collected in dry flasks. The refractive index of the filtrate from each sample was then determined, employing a Pulfrich refractometer and a sodium flame as the source of light. Denoting the refractive index of the filtrate from any given sample by n and that of the pure solvent (1.35 per cent HCl) by n_1 , the quotient $\frac{n - n_1}{0.00172}$ is the number of grams of salmine dissolved in 100 cc of the solvent at the moment when the sample was abstracted.¹

The relationship which was found to subsist between the period of extraction and the mass of salmine extracted is displayed graphically in the accompanying figure. It will be seen that the rate of extraction is at first very great, but that it very rapidly falls off



It does not fall to zero, however, in other words, the curve does not approach an asymptote, in which respect it differs very strikingly

¹ Brailsford Robertson *This Journal*, vi, p. 307, 1912

from the curve which depicts the progress of a chemical reaction or of the solution of a crystalloid

This is the type of relationship which I have previously found to subsist between the mass of casein dissolved by dilute alkali and the period during which the casein is in contact with the solvent² It is the relationship which Cameron and Bell³ and, later, Ostwald and Goppelsroeder⁴ found to subsist between the amount of fluid absorbed by a column of sand or of a strip of filter paper and the time during which the fluid has remained in contact with a portion of its surface This relationship may be expressed by the formula $x = Kt^m$, where x is the amount of fluid absorbed (or casein dissolved, or salmine extracted) and t is the time, K and m being constants In the accompanying table the values of x found in the above experiment and those calculated from the formula are compared, the constants K and m being determined from all of the observations by the method of least squares, employing for this purpose the form

$$\log_{10} x = m \log_{10} t + \log_{10} K$$

The possible experimental error in the determination of the concentration of a salmine solution by means of its refractive index (due to an error of $\pm 1'$ in reading the angle of total reflection) is ± 0.05 gram per 100 cc It will be seen that the differences ($=\Delta$) between the observed and calculated values of x are considerably less than the possible error in the determination of the concentration of the salmine in the filtrates

The extreme rapidity with which the salmine leaves the tissue in the first few moments of the extraction and the slowness with which the remaining amount is extracted, very vividly remind one of the extreme velocity with which, during the first instants, a liquid mounts a capillary tube and of the exceedingly slow "creeping" of the liquid up the sides of the tube which is still

² T Brailsford Robertson *Journ of Physical Chem*, xiv, p 377, 1910

³ Cameron and Bell Bulletin No 30, p 50 Bureau of Soils, U S Dept of Agriculture, 1905, *Journ of Physical Chem* x, p 658, 1906

⁴ Wo Ostwald *Zeitschr f Kolloidchemie* (2 Supplementheft) 1908, F Goppelsroeder *Verhandl naturforsch Gesellsch zu Basel* xix, Heft 2, 1907

Analysis
Results are expressed

OBSERVERS	FRENCH ⁴		GORUP-BESANEZ ⁵		HOPPE SEYLER ⁶
Source	Bladder bile		Bladder bile		Bladder bile
Bile salts	72.2	91.4	107.9	56.5	39.0
Mucin and pigment	26.6	29.8	22.1	14.5	12.9
Cholesterol	1.6	2.6	47.3	30.9	3.5
Fat	3.2	9.2			7.3
Soaps					13.9
Lecithin					5.3
Total solids	140.0	148.0	177.3	101.9	
Inorganic	6.5	7.7	10.8	6.3	37.6
Water	860.0	859.2	822.7	898.1	
Fatty acids					

OBSERVERS	COPEMAN & WINSTON ¹²	YEO & HERROUN ¹³	ROBSON ¹⁴	PATON & DALFOUR ¹⁵	
Source	Biliary fistula	Biliary fistula (cancer)	Biliary fistula	Biliary fistula	
Bile salts	6.3	2.2	7.6	4.0	3.5
Mucin and pigments	2.5	1.5	1.3	7.1	4.6
Cholesterol	1.0	0.38	0.45	0.53	0.75
Fat			0.12	0.09	
Soaps			0.97	0.15	
Lecithin					
Total solids	14.23	12.8	18.0	11.9	15.3
Inorganic	4.5	8.8	7.6		6.4
Water	985.6	987.2	981.9	988.0	984.8
Fatty acids					

* Six analyses of bladder bile from children of the following ages: 1 day, 1 month, 2 months, 5 months, 9 months, 1 year. Figures in this column represent minimum and maximum values.

† Includes lecithin and fatty acids.

⁴ *Ann f d ges Heilk*, v, p 42, 1845

⁵ *Vierteljahreschr f prakt Pharmakol*, III, p 86, 1851

⁶ Hoppe-Seyler *Physiologische Chemie*, 1877-1881, pp 299 and 301

¹² *Journ of Physiol*, x, p 213, 1889

¹³ *Ibid*, v, p 116, 1884

¹⁴ *Proc of Roy Soc*, XLVII, p 499, 1890

¹⁵ *Laboratory Reports of Royal College of Physicians, Edinburgh*, III, p 191,

Human bile
parts per 1000 (by weight)

JACOBSEN ⁷		TRIFANOWSKI ⁸		HAMMARSTEN ⁹			BAOINSKY & SOMMER FELD ¹⁰	BIRCH & SPONG ¹¹
Bladder bile	Bladder bile various diseases	Bladder bile	Liver bile			Bladder bile children	Bladder bile	
10 1	28 0	19 6	9 31	18 2	9 04	25 2	0	
2 3	24 8	13 0	5 29	4 29	5 15	20 0	12 1	
0 6	2 5	3 3	0 63	1 6	1 5	3 4		
0 1	5 2†	3 6	0 22†	0 57	0 65	6 7		
1 4	8 2	16 3	1 23†	1 36†	1 01†			
0 05		0 17		0 57	0 65			
22 6	91 2	89 2	25 2	35 3	25 4	103 5	20 3	
5 78						9 1	8 2	
977 4	909 0	911 0	974 8	964 7	974 6	896 5	979 7	

HAMMARSTEN ⁹		ZEBROW-SKI ¹⁴	MAJEWSKI & ZEBROW-SKI ¹⁵	BONNANI ¹⁶	JACOBO-WITSCH ¹⁷	MENZIES ²⁰	ROSEN-BLOOM
Bladder bile		Biliary fistula	Bladder bile	Biliary fistula	Bladder bile (infants)*	Biliary fistula	Biliary fistula
97 0	87 0	34 3	167 6	18 33	5 5-14 0	4 2	10 1
41 9	44 4	11 14	23 12	4 97	9 0-36 0	9 3	4 86
9 86	8 7	5 16†	21 8†	1 67	1 7-3 0	0 94	2 61†
1 9	6 5			0 96	2 5-9 8†	2 98†	6 85
11 2§	10 6§		24 7				2 6
2 23	1 41			0 58			6 42
170 3	160 2	112 2		35 44	98-145	22 5	29 8
		61 3	88 8	7 2	5 2-7 3	5 8	9 2
829 7	839 8	887 8		964 6	855-900	974 5	970 2
				1 37	0 7-1 0		1 2

† Fatty acids from soaps

§ Includes fatty acids

¶ This specimen contained a trace of cholesterol esters

⁷ *Ber d deutsch chem Gesellsch*, vi, p 1026, 1873

⁸ *Pflüger's Archiv*, ix, p 492, 1874

⁹ *Textbook of Physiological Chemistry*, 1911, p 413

¹⁰ *Verhandl d physiol Gesellsch zu Berlin*, 1894-95, *Arch f Physiol*, 1895, p 562

¹¹ *Journ of Physiol*, viii, p 378, 1887

¹² *Maly's Jahresbericht*, xxxi, p 546, 1901

¹³ *Ibid*, xxvii, p 505, 1902

¹⁴ *Ibid*, xxvii, p 508, 1902

¹⁵ *Jahrb f Kinderheilk*, xxiv, p 373, 1886

²⁰ *Biochem Journ*, vi, p 210, 1912

THE BEHAVIOR OF SOME HYDANTOIN DERIVATIVES IN METABOLISM II

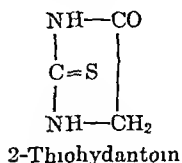
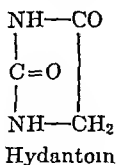
2-THIOHYDANTOINS

By HOWARD B LEWIS

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(Received for publication, February 11, 1913)

In a former paper,¹ it has been shown that the hydantoin nucleus is excreted unchanged in the urine, when introduced into the organism of the cat, rabbit or dog. The present study deals with the behavior of some thiohydantoin in which sulphur replaces the oxygen in the 2-position of the hydantoin nucleus



The behavior of compounds containing this type of sulphur linkage is especially interesting in view of the recent studies of Johnson and his co-workers.² In these the probable existence of sulphur in the protein molecule in forms other than as cystine or cysteine groupings is discussed, and the possible occurrence of thio-amide sulphur, $-\text{NHCS}-$, corresponding to the acid amide form, $-\text{NHCC}-$, present in polypeptides is suggested. This type of replacement of oxygen by bivalent sulphur is well represented in the thiohydantoin. It must be pointed out, however, that the $-\text{CS}-$ group attached to two nitrogen atoms as in thiohydantoin is much

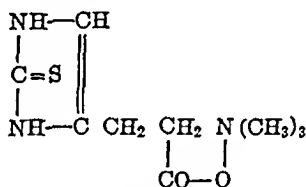
¹ This *Journal*, xii, pp 347-56, 1912

² Johnson this *Journal*, ix, pp 331-2, 439-48, 449-63, 1911, vii, pp 175-96, 1912

more stable than the $-CS-$ group placed between a nitrogen and a carbon atom as in a thioamide, $R-CS-NH_2$, or as in dithiopiperazine, the anhydride of the thiopolyptide, recently prepared by Johnson and Burnham³ In the latter compound the sulphur may be readily split off as hydrogen sulphide by boiling with hydrochloric acid

Since thiohydantoin contains the thioamide type of sulphur grouping, a study of the ability of the organism to oxidize and excrete the sulphur of these compounds should throw some light on the possible behavior of similar sulphur groupings, whose presence in the protein molecule has been suggested

It has recently been shown that a compound which closely resembles the thiohydantoin may be obtained from the ergot of rye Barger and Ewins⁴ have found that the base *ergothioneine* isolated from ergot by Tanret⁵ is probably the betaine of α -amino- β -2-thioglyoxaline-4 (or 5)-propionic acid



Ergothioneine

The simplest compound which contains this type of sulphur combination is thiourea, $NH_2-CS-NH_2$, the behavior of which has repeatedly been the subject of study⁶ Thiourea is non-toxic, is excreted unchanged in the urine, and does not increase the oxidized sulphur content of the urine After the administration of thiourea to rabbits, Pohl⁷ reports the elimination of an alkyl

³ Johnson and Burnham *this Journal*, ix, pp 449-63, 1911

⁴ Barger and Ewins *Journ of Chem Soc* (London), xcix, pp 2336-41, 1911

⁵ Tanret *Journ de pharm et de chim*, xxx, pp 145-53, 1909, *Compt rend de l'Acad des Sci*, cxlix, pp 222-24, 1909

⁶ Cf Lange Inaugural Dissertation, Rostock, 1892, *Jahresber u d Fortsch d Thierchem*, xxii, p 67, 1892, Sato *Zeitschr f physiol Chem*, lxi, pp 378-96, 1909, Masuda *ibid*, lxxvii, p 28, 1910

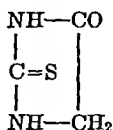
⁷ Pohl *Arch f exp Path u Pharm*, li, pp 341-45, 1904.

sulphide, probably ethyl sulphide, in the breath. No sulphide could be detected in the urine. The greater part of the thiourea was excreted unchanged and only a few milligrams of alkyl sulphide were obtained from the expired air.

In the present work, two preliminary experiments in which 2 grams of thiourea were injected subcutaneously into rabbits were made. In confirmation of the work of Pohl, a peculiar garlic-like odor was observed in the breath five hours after the injection, but no alkyl sulphide could be obtained from the urines by the method of Abel.⁸ The total sulphate-sulphur content of the urine was unchanged, while the "neutral sulphur" was increased in proportion to the amount of sulphur injected as thiourea. The urines of the experimental days gave strong reactions with potassium ferrocyanide and acetic acid, as described for thiourea by Sato.⁹ No toxic effects were apparent.

In the experiments to be recorded with the thiohydantoin the animals used were, with one exception, rabbits, which were maintained on a uniform diet of carrots and oats. The urine was collected from the bladder by gentle pressure at the same hour daily. The substances, when fed, were dissolved in water and introduced through a stomach tube. The routine analytical procedures included the Kjeldahl-Gunning method for total nitrogen, Folin's method for total sulphate-sulphur, and Benedict's methods¹⁰ for urea and total sulphur. "Neutral sulphur" was obtained by difference. It is of interest to note that all of the compounds studied give the color reaction with phosphotungstic acid and sodium carbonate described by Folin for uric acid.

2-Thiohydantoin



⁸ Abel *Zeitschr f physiol Chem*, xx, pp 253-78, 1895

⁹ Sato *Biochem Zeitschr*, xxiii, pp 45-6, 1910

¹⁰ S R Benedict *this Journal*, vi, pp 363-71, 1909 (total sulphur), *ibid*, viii, pp 405-22, 1910 (urea)

This compound was prepared from hippuric acid and ammonium thiocyanate as described by Johnson and Nicolet¹¹ Its purity was established by its melting point (226-7°) and a Kjeldahl nitrogen determination (24.12 per cent N) The compound had a slight red-yellow color and when dissolved in water gave a yellowish solution With picric acid and an alkali a color resembling that given by creatinine in Jaffé's test was obtained

The substance proved to be toxic for rabbits, doses of 0.5 gram causing death within twelve hours The most noticeable symptoms were loss of muscular control and power of co-ordination, dyspnoea and convulsions The degree of toxicity is shown by the following illustrative protocol and the accompanying table (I) which summarizes all the results obtained

Rabbit 14 Weight 1.4 kgs 8.30 a.m. Received 0.5 gram of 2-thio hydantoin subcutaneously Resumed eating on return to cage

9.00 Has stopped eating

10.00 Apparently normal

11.00 Apparently normal except for slightly increased respiration

12.00 Restless Poor control of hind legs Tremors Rapid respiration

1.00 Lack of co-ordination Control of hind legs lost Dyspnoea

2.00 An occasional convulsion

2.55 More quiet Convulsions less frequent Dyspnoea

3.10 Urinates Urine deep orange red

3.45 Violent convulsions, dyspnoea Pupils very greatly dilated

4.00 Dead

Autopsy Muscles stained yellow red at point of injection Fluid all absorbed Viscera appear normal Bladder empty Lungs and heart congested

Examination of Urine Color deep orange red Jaffé's picric acid test very brilliant Albumin test negative Reduction test with Benedict's solution gives a black precipitate of copper sulphide

¹¹ Johnson and Nicolet *Journ Amer Chem Soc*, **xxiii**, p 1973, 1911,
Johnson *Amer Chem Journ*, **xli**, pp 68-9, 1913

TABLE I
Toxicity of 2-Thiohydantoin

ANIMAL	WEIGHT	DOSE PER KGM	METHOD OF ADMINISTRATION	RESULTS
	<i>kms</i>	<i>grams</i>		
Rabbit 12	1 70	1 17	<i>per os</i>	Death in 3½ hours
Rabbit 13	1 67	0 59	<i>per os</i>	Death in 4½ hours
Rabbit 14	1 40	0 31	subcutaneously	Death in 7½ hours
Rabbit 15	1 58	0 125	subcutaneously	Death in 24 hours
Rabbit 16	1 48	0 066	subcutaneously	Refused food for 2 days Restless Recovered
Rabbit 19	1 80	0 277	subcutaneously	Preparation 2 * Death in 7½ hours
Rabbit 22	1 92	0 260	subcutaneously	Preparation 3 * Death 9½ hours
Rabbit 27	1 78	0 056	subcutaneously	Recovery Refuses food for two days
Rabbit 28	1 63	0 061	subcutaneously	Recovery Refuses food for two days
Rabbit 29	1 70	0 088	subcutaneously	Recovery Refuses food for two days
Cat C	3 50	0 157	<i>per os</i>	Death in 8 hours

* After the earlier experiments had shown the marked toxicity a new preparation 2 was prepared and especially purified. Preparation 3 was a preparation used in experiments with rabbits 19, 22, 27 and 29 and cat C especially purified by Mr Ben H. Nicolet to whom I am indebted for this and other assistance.

The toxicity of this compound must be attributed to the sulphur which replaces the oxygen in the hydantoin nucleus. While approximately 0.125 gram per kilo body weight is the lethal dose for the thiohydantoins, amounts of over 1.5 grams of hydantoin per kilo have been fed to rabbits without any toxic effects.

It was first planned to study the distribution of sulphur in the urine after the administration of the thiohydantoin in order to note any oxidation of the sulphur. But the relatively small size of the dose which is necessary to prevent a fatal outcome of the experiment made such a study unpromising. An attempt was made in the case of rabbits 22, 27, 28, 29, and cat C to identify the unchanged thiohydantoin in the urine.

Many attempts to isolate the thiohydantoin as such were unsuccessful. At length a method of procedure was adopted which, while it gave positive evidence of the presence of unchanged thiohydantoin in the urine, did not furnish absolute proof. Thio-

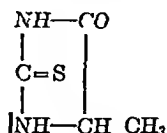
hydantoin when boiled with chloroacetic acid are desulphurized¹² with the formation of hydantoin and thioglycollic acid. The latter acid may be detected by a delicate color reaction described by Heffter.¹³ A very dilute solution of thioglycollic acid gives, on the addition of sodium nitroprusside and an alkali, a purple-red color, changing quickly to brown-red and soon disappearing. This reaction is also given by the alkyl sulphides, ethyl and benzyl mercaptans, cysteine, α - and β -thiolactic acids and thiophenol.

The urines were first tested for this reaction before treatment with chloroacetic acid to rule out the presence of the alkali sulphides and the other above-mentioned compounds which give this reaction. In no case was any purple color obtained from the dilute urine. The urine was then concentrated on the water bath to small volume, 2-3 grams of chloroacetic acid added, and the mixture boiled with a return condenser from four to six hours. The contents of the flask were cooled, treated with animal charcoal to decolorize, and the color reaction carried out. In all the experimental urines the reaction was positive. Normal urines which were treated in the same manner never gave positive reactions. To avoid the color of the urine, which interfered with a delicate test, the liquid after boiling with chloroacetic acid was in some cases evaporated to dryness and extracted with ether, in which thioglycollic acid is soluble. The ether was then removed by evaporation, the residue taken up in water, and the color reaction carried out. The reactions were more brilliant when the test was performed in this way.

While the possibility that there may be other compounds formed in the body, which yield thioglycollic acid on boiling with chloroacetic acid, is not entirely excluded, the demonstration of the presence of thioglycollic acid after the chloroacetic acid treatment in the experimental urines gives a strong indication of the presence of unchanged thiohydantoin. 2-Thiohydantoin, like hydantoin, is eliminated probably unchanged by the rabbit. Unlike hydantoin it is very toxic for rabbits, 0.125 gram per kilo being a lethal dose.

¹ Johnson, Pfau and Hodge *Journ Amer Chem Soc*, xxxiv, pp 1041-48, 1912

¹³ Heffter *Medizin-naturwiss Archiv*, I, p 81, 1908

2-Thio-4-methylhydantoin

2-Thio-4-methylhydantoin was prepared by the action of ammonium thiocyanate on alanine as described by Johnson¹⁴ Its purity was shown by its melting point (161°) and a Kjeldahl nitrogen determination (21.53 per cent N)

2-Thio-4-methylhydantoin was toxic for rabbits although far less so than the 2-thiohydantoin The lethal dose was 0.6 gram per kilo body weight, or more than three times that of 2-thiohydantoin The symptoms were very similar to those produced by the 2-thiohydantoin, although the convulsions were less severe than with the latter Most marked was the intense albuminuria in all those cases in which the fatal dose was given A study of the typical protocol given below, together with the tabular summary (table II), will best serve to illustrate the toxicity

Rabbit 34 Weight 1.7 kilos Dec 12, 8:30 a.m. Received 1 gram of 2-thio-4-methylhydantoin subcutaneously

11:00 Drowsy Sits with eyes closed

12:00 Tremors Runs excitedly around cage when roused

3:00 Very excitable Runs around cage Occasional convulsions

5:00 Quiet

December 13 8:00 a.m. Drowsy Urine deep yellow Protein tests positive (Heller's, picric acid, heat coagulation) No casts

Animal quiet all day Ate no food

Dec 14 8:00 a.m. Animal dead in cage Body still warm Urine, protein test strongly positive

Autopsy revealed nothing abnormal

¹⁴ Johnson *this Journal*, vi, pp 97-101, 1912, *Amer Chem Journ*, xlix, pp 68-9, 1913

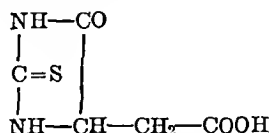
TABLE II

Toxicity of 2-Thio-4-Methylhydantoin

ANIMAL	WEIGHT	DOSE PER KGM	METHOD OF ADMINISTRATION	RESULTS
	<i>kgs</i>	<i>grams</i>		
Rabbit 20	2 1	0 25	<i>per os</i>	No symptoms
Rabbit 21	1 9	0 25	subcutaneously	No symptoms
Rabbit 20	2 1	0 50	<i>per os</i>	Restless Refuses food for two days
Rabbit 31	1 6	0 93	subcutaneously	Death in 31 hours Albumin test positive
Rabbit 33	1 2	0 83	subcutaneously	Death in 1½ hours
Rabbit 31	1 7	0 58	subcutaneously	Death in 2 days Albumin test positive
Rabbit 35	1 8	0 69	<i>per os</i>	Death in 5½ hours Albumin test positive
Rabbit 36	1 7	0 58	<i>per os</i>	Death in 3 days Albumin test negative

No attempt was made to identify the unchanged thiohydantoin in the urine

The chief interest in the above experiments lies in the lowered toxicity of the sulphur hydantoins due to the substitution of a methyl group for one hydrogen in the 4-position

2-Thiohydantoin-4-acetic acid

This hydantoin was prepared from ammonium thiocyanate and asparagine as described by Johnson and Guest¹⁵. A determination of its melting point (222°) and nitrogen content (16.09 per cent N) showed its purity. This compound is rather difficultly soluble in water, hence in the experiments in which it was used sodium carbonate was added to form the sodium salt which is more soluble.

¹⁵ Johnson and Guest *Amer. Chem. Journ.*, xlviii, pp 108-9, 1912, Johnson *ibid.*, xlix, pp 68-9, 1913

After several preliminary experiments had shown that 2-thiohydantoin-4-acetic acid was non-toxic for rabbits and that as large a dose as 2 grams had no obvious effects on the animal, a study of the sulphur elimination and distribution in the urine was made.

Rabbit 30 Daily diet, 250 grams of carrots and 25 grams of oats. This was completely consumed. On the day of the injection the animal showed no symptoms, except a refusal to eat for several hours. On the fourth day, a subcutaneous injection of 2 grams of 2-thiohydantoin-4-acetic acid was made (N content = 0.321 gram, S content = 0.368 gram). The protocol follows.

Rabbit 30, Weight, 1.69 kgs

DATE	VOLUME	SPECIFIC GRAVITY	TOTAL N	UREA + NH ₃ -N	UREA + NH ₃ -N	N NOT UREA + NH ₃ -N	TOTAL S	TOTAL SO ₄ S	NEUTRAL S	TOTAL SO ₄ S	NEUTRAL S
					Total N						
	cc		gram	gram	per cent	gram	gram	gram	gram	per cent	per cent
1	85	1.040	0.675	0.555	82.2	0.120	0.0667	0.0575	0.0092	86.2	13.8
2	125	1.020	0.720	0.615	85.4	0.105	0.0642	0.0536	0.0106	81.9	18.1
3	125	1.030	0.570	0.465	81.7	0.105	0.0629	0.0497	0.0132	79.0	21.0
4	100	1.040	1.088	0.735	67.5	0.353	0.3478	0.0306	0.3172	8.8	91.2
5	125	1.023	0.705	0.570	80.8	0.135	0.0567	0.0371	0.0196	65.4	34.6

Rabbit 40 Diet, 25 grams of oats and 250 grams of carrots. This diet was consumed as usual on the day of the administration of the 2-thiohydantoin-4-acetic acid. On the fourth day 1.75 grams of 2-thiohydantoin-4-acetic acid were given *per os* (S content = 0.322 gm). No toxic symptoms were noted. The protocol follows.

Rabbit 40 Weight 1.82 kgs

DATE	VOLUME	SPECIFIC GRAVITY	TOTAL S	TOTAL SO ₄ S	NEUTRAL S	SO ₄ S	NEUTRAL S
	cc		gram	gram	gram	per cent	per cent
1	100	1.015	0.0718	0.0561	0.0157	78.1	21.9
2	180	1.014	0.0767	0.0566	0.0201	74.8	26.2
3	150	1.020	0.1167	0.0809	0.0358	69.3	30.7
4	160	1.023	0.2824	0.0581	0.2244	20.5	79.5
5	80	1.036	0.1596	0.0790	0.0806	49.5	50.5
6	160	1.015	0.0980	0.0691	0.0289	70.5	29.5
7	160	1.019	0.0946	0.0728	0.0218	77.0	23.0

In a third experiment in which 1.75 grams of 2-thiohydantoin-4-acetic acid were given *per os* to a rabbit of 1.5 kilos, similar results were obtained, the "neutral sulphur" rising from a preliminary average of 23.2 per cent to 88.8 per cent on the day of the injection.

In these three experiments on rabbits there was no evidence of an oxidation of the sulphur by the organism, a reaction which should result in an increased total sulphate-sulphur elimination. In no case was the sulphate-sulphur increased on the day of the injection. In each instance, however, the "neutral sulphur" elimination was increased in proportion to the amount of sulphur given as 2-thiohydantoin-4-acetic acid. No attempt was made to recover the unchanged hydantoin from the urine.

It is interesting to note that these results, which show that the sulphur in this type of combination is not oxidized by the organism, are in agreement with the results obtained by Steudel¹⁶ and Mendel and Myers¹⁷. The former working with 2-thio-4-methyluracil found that it was excreted unchanged by the organism of the dog. The latter studied the distribution of sulphur in the urine after the administration of 2-thiouracil to rabbits and found no increase in the oxidized sulphur, but a marked increase in the "neutral sulphur" of the urine. These findings all agree in demonstrating the stability of thioamide sulphur in the organism.

Experiments on rabbits in which doses of 0.44 and 0.79 gram per kilo of 2-thiohydantoin-4-acetamide were given *per os* to rabbits demonstrated that in such amounts this substance is non-toxic for rabbits.

The lack of toxicity of 2-thiohydantoin-4-acetic acid for the rabbit would seem to indicate that the substitution of alkyl groups or their oxidation products for a hydrogen in the 4-position diminishes the toxicity shown by the sulphur in 2-thiohydantoin. The increase in the molecular weight of the substituted alkyl group, in the substances studied at least, gradually decreases the toxicity. Thus the lethal dose of the unsubstituted thiohydantoin was found to be about 0.125 gram, the substitution of a methyl group raised the fatal dose to 0.6 gram, while substitution of an acetic acid or acetamide group caused a loss of toxicity. It is of interest to know whether this theory of decreased toxicity will be confirmed by a

¹⁶ Steudel *Zeitschr f physiol Chem*, **xxix**, pp 136-42, 1903

¹⁷ Mendel and Myers *Amer Journ of Physiol*, **xxvi**, pp 77-105, 1910

study of the effect of other radicals, substituted both in the 4-position as in the compounds studied and in other positions in the ring. An investigation of the effects of such substitution is proposed.

SUMMARY

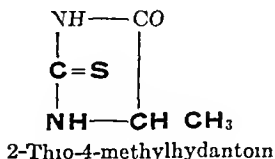
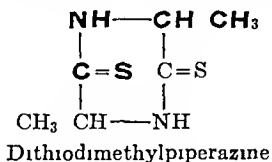
2-Thiohydantoin is toxic for rabbits. The substitution of an alkyl group in the 4-position decreases the toxicity. 2-Thio-4-methylhydantoin is less toxic than 2-thiohydantoin, while 2-thiohydantoin-4-acetic acid is not toxic in doses of 2 grams. 2-Thio-4-methylhydantoin in fatal doses causes an albuminuria in rabbits. The sulphur contained in 2-thiohydantoins is not oxidized in the organism of the rabbit, but is excreted probably unchanged.

I wish to acknowledge my indebtedness to Professor Lafayette B. Mendel under whose direction this work has been carried out and to Professor Treat B. Johnson who has aided in the questions of organic chemistry involved.

ADDENDUM

Through the courtesy of Professor Treat B. Johnson, a study of one of the thiopolypeptides already referred to was made possible. The compound with which the experiments were carried out was *dithiodimethylpiperazine*, the preparation and properties of which will be described in a later paper from Professor Johnson's laboratory. The new compound is very insoluble in water and was administered as the sodium salt. It was first suspended in water, sodium hydroxide added in the cold until solution took place, and the whole immediately neutralized. These precautions were made necessary because of the ease with which *dithiodimethylpiperazine* splits off sulphur in the presence of free alkali. As has already been pointed out, this thioamide type of sulphur combination is very unstable.

The relation of this thiopolypeptide derivative to the thiohydantoins which have been shown to be toxic may be seen from a comparison of the structural formulae



Two $-CSNHCHCH_3$ groupings make up the thiopolypeptide derivative. This same grouping occurs once in the thiohydantoin. Hence from a theoretical standpoint, the toxicity of the thiopiperazine derivative should be greater than that of the thiohydantoin. This was found to be the case. Two typical protocols follow.

Rabbit 43 Weight 1.70 kgms 10 07 a m Received 0.3 gram (= 0.176 gram per kilogram body weight) dithiodimethylpiperazine, prepared as described above, *subcutaneously*

10 25 Tremors Very restless Dyspnoea

10 30 Slight convulsions

10 35 Dyspnoea very marked Animal gasps, gnashes teeth

10 40 Tremors more marked Pupils widely dilated

10 45 Animal has a very violent convulsion in the course of which it forces open the cover of the cage. Immediately following tetanic symptoms, not clonic, appear.

10 55 Convulsions very frequent. As animal was apparently dying ether was given to neutralize the effects of convulsions. Animal became quiet immediately and on being allowed to recover from the anesthesia showed convulsions again.

12 05 The animal had been etherized for more than an hour, but the increasing violence and frequency of the convulsions made deeper anesthesia constantly necessary. The animal was now allowed to recover from anesthesia. Immediately, extreme dyspnoea, labored breathing and convulsions began.

12 35 Very weak

1 15 Dead

Autopsy Nothing abnormal. Urine trace of albumin.

Rabbit 45 Weight 1.8 kgms 9 00 a m Received 0.3 gram dithiodimethylpiperazine prepared as described above *per os*

9 07 Tremors

9 10 Convulsions

9 15 Lies on side in convulsions

9 23 Extreme dyspnoea

9 33 Very weak

10 00 Death

Autopsy Nothing abnormal except congested heart and lungs

The close parallelism between the symptoms above described and those produced by alkali sulphides or hydrogen sulphide immediately suggests that an explanation of the toxicity may be found in the liberation of sulphides in the organism from the unstable sulphur linkage. Further investigations on this substance are in progress.

ON CEREBRONIC ACID

SECOND PAPER

By P A LEVENE AND C J WEST

(From the Rockefeller Institute for Medical Research, New York)

(Received for publication, February 19, 1913)

On the basis of experiments published in a previous communication Levene and Jacobs¹ reached the view that cerebronic acid had the structure of an α -hydroxypentacosanic acid. On reduction the acid formed a hydrocarbon melting at 54°-57°C. The melting point for the normal pentacosan, according to Krafft and Marie, should be 53.5°-54°. Hence it remained uncertain whether the discrepancy in the melting points was due to the fact that the hydrocarbon obtained from cerebronic acid contained some impurity or to a structural difference in the normal pentacosan and in the hydrocarbon obtained from cerebronic acid. It was, therefore, concluded to prepare a larger quantity of the material, which would permit of a more perfect purification of the hydrocarbon.

This was accomplished with the result that on reduction of cerebronic acid a hydrocarbon was obtained that melted at 53°-54°C, which harmonizes with the melting point corresponding to the normal pentacosan.

The normal nature of the carbon chain of cerebronic acid was corroborated further by the fact that the acid, $C_{24}H_{48}O_2$, obtained on the oxidation of the former was reduced to a hydrocarbon melting at 51°-52°C, and that Krafft found 51.1°C to be the melting point of the normal tetracosan. Hence, it may be considered proven that cerebronic acid is a normal α -hydroxypentacosanic acid.

In course of the present investigation great care was taken in establishing the exact condition for preparation of the most important derivatives of the acid, also of the methods of separation and of purification of the acid.

¹ This *Journal*, xii, p 381, 1912

EXPERIMENTAL PART

Preparation of cerebronic acid

The mixture of cerebronic acid and ester obtained from the hydrolysis of cerebrine² is heated with an excess of alcoholic sodium hydroxide for four hours, during which most of the sodium salt separates out. The mixture is cooled, the soaps filtered off and washed with methyl alcohol and ether. The sodium salt is then recrystallized from boiling ethyl or methyl alcohol. The cerebronic acid is liberated by suspending the salt in dilute hydrochloric acid and heating on the water bath until the free acid has completely melted. When cold this is filtered off, recrystallized from alcohol and the excess of alcohol removed by melting in vacuum on the water bath. The product thus obtained is pure enough for most operations. It will not, however, give sharp values when titrated to determine the molecular weight. The acid used for this purpose must be further purified through the lead salt. The hot methyl alcoholic solution of the acid is treated with a hot solution of lead acetate in the same solvent as long as a precipitate forms. Finally a few drops of concentrated ammonia are added to neutralize the free acetic acid. After being cooled the lead salt is filtered off and washed with a little warm methyl alcohol. It is then suspended in warm, dry toluene and decomposed by treating with hydrogen sulphide for two hours. The mixture should be kept warm on a water bath and constantly stirred during this time. The lead sulphide is allowed to settle, the toluene solution of the acid filtered off, the sulphide washed with warm toluene and the filtrate concentrated in vacuum. The colorless residue is then recrystallized from absolute alcohol and the excess of alcohol removed on the steam bath. If ammonia is not added in the precipitation of the lead salt the sulphide may come down in a colloidal form. In this case, the entire mixture is concentrated in vacuum and the acid extracted from the residue with a large quantity of boiling alcohol.

The acid thus prepared melted at 77°–80° and, therefore, consisted principally of the inactive form. When fractionated with lithium acetate in methyl alcohol into three fractions, each fraction,

² Levene and Jacobs *loc cit*, p. 383

before recrystallization, melted at 76° – 77° and, when recrystallized from petroleum ether, melted at 83° – 84° . This is the melting point found for the inactive form and reported in a previous paper

0.1200 gram of the substance gave 0.3330 gram CO_2 and 0.1350 gram H_2O

1.7115 grams of the acid dissolved in a mixture of benzene and pure methyl alcohol required 43 cc of $\frac{N}{10}$ NaOH for neutralization, using phenolphthalein as an indicator

0.4802 gram of the acid, as above, required 12.15 cc of $\frac{N}{10}$ NaOH for neutralization

	Calculated for $\text{C}_{21}\text{H}_{30}\text{O}_4$	Found
C	75.33	75.68
H	12.50	12.59
M W	398	398 395

Salts of cerebronic acid

Salts The sodium and lithium salts were prepared and their solubilities studied in order to find the best solvent for purification and also to establish the conditions for the separation of the oxidation product of cerebronic acid from the unchanged acid (see below). The determinations were carried out as follows. The salt was treated with an excess of the boiling solvent, the saturated solution was filtered off, using a hot funnel, and allowed to stand in the ice box over night. The precipitate was filtered off, dried and weighed. The residue from the filtrate was also determined. This gave the total solubility, the amount that could be obtained in recrystallization and the solubility at 0° .

Sodium salt³ **SOLUBILITIES** 140 cc of boiling methyl alcohol, saturated with the sodium salt, deposited, upon cooling to 0° , 1.47 grams. 20 cc of the filtrate, saturated at 0° , gave a residue of 0.09 gram upon evaporation to dryness.

110 cc of ethyl alcohol, as above, deposited 2.094 grams. 20 cc of the filtrate gave a residue of 0.032 gram.

Therefore, 100 cc of boiling methyl alcohol takes up 1.50 grams of the sodium salt. At 0° it contains 0.45 gram.

100 cc of boiling ethyl alcohol contains 2.06 grams, at 0° , 0.16 gram.

³ Thierfelder *Zeitschr f physiol Chem*, xliii, p 26, 1904-05

ANALYSIS 0.1125 gram of the substance gave 0.2931 gram CO_2 and 0.1196 gram H_2O

	Calculated for $\text{C}_{23}\text{H}_{36}\text{O}_7\text{Na}$	Found
C	71.19	71.06
H	11.72	11.90

Lithium salt SOLUBILITIES 100 cc of boiling methyl alcohol takes up 3.46 grams of the salt, while at 0° it contains 0.235 gram. 100 cc of ethyl alcohol at its boiling point takes up 1.42 grams of the salt and at 0° contains 0.39 gram.

ANALYSIS 0.1448 gram substance gave 0.3928 gram CO_2 and 0.1593 gram H_2O

	Calculated for $\text{C}_{23}\text{H}_{36}\text{O}_7\text{Li}$	Found
C	74.18	74.00
H	12.21	12.31

Inactive ethyl ester

Thudichum⁴ describes an ethyl ester of neurostearic acid (cerebronic acid) which he obtained from the hydrolysis of phrenosine (cerebrine) with ethyl alcohol and sulphuric acid. This product melted at 56° but when distilled in vacuum melted at 52° . Since the acid derived from this melted at 84° it must have been the inactive ester. We prepared the ester from the inactive acid as follows: 10 grams of the acid were dissolved in 500 cc absolute ethyl alcohol, 10 cc concentrated sulphuric acid added and the mixture heated on the water bath for seven hours. Upon standing over night at 0° the ester almost completely separates out. This was recrystallized from alcohol containing sulphuric acid, then from absolute alcohol and finally from dry acetone. It is very soluble in ethyl acetate, from which it separates in large glistening crystals. The ester melted at 52° – 53° and solidified at 51° – 52° . For analysis the product was dried three hours in a chloroform bath over sulphuric acid.

0.1128 gram substance gave 0.3142 gram CO_2 and 0.1265 gram H_2O

	Calculated for $\text{C}_{23}\text{H}_{36}\text{O}_7, \text{C}_2\text{H}_5$	Found
C	75.79	75.98
H	12.55	12.75

⁴ Thudichum *Chemical Constitution of the Brain*, p. 162, German edition, p. 195.

Acetate of the ethyl ester, $C_{23}H_{47}CH(OCOCH_3)CO_2C_2H_5$

Two grams of the ethyl ester were dissolved in 40 cc of acetic anhydride and heated to gentle boiling for an hour. Upon cooling the reaction product crystallized out. This was recrystallized from acetone and twice from petroleum ether. It forms colorless crystals, which melt at 55° – 57° and solidify at 53° – 55° . It was dried in the chloroform bath for analysis.

0.1336 gram of substance gave 0.3630 gram CO_2 and 0.1457 gram H₂O

	Calculated for $C_{23}H_{46}O_4$	Found
C	74.28	74.16
H	12.05	12.19

Marie⁵ describes a similar compound which was obtained by the action of lead acetate upon the ethyl ester of bromocerotic acid and to which he ascribes the above formula. This melted at 57° – 58° . Since then it has been claimed that cerotic acid contains twenty-six carbon atoms, if this is so, then the two compounds are not identical. The question will be investigated further.

Inactive methyl ester

Thuerfelder⁶ describes a methyl ester of cerebronic acid, which he obtained in the hydrolysis of cerebrine and which gave upon hydrolysis an acid melting at 100° – 101° . This ester melted at 65° , and was probably either the active form or a mixture of the active with the inactive. The inactive ester was prepared by boiling a solution of 5 grams of the inactive acid in 500 cc of absolute methyl alcohol, which contained 5 cc of concentrated sulphuric acid, for five hours. The ester which separated out upon standing over night was recrystallized from methyl alcohol, petroleum ether and finally acetone and formed colorless crystals, which melted at 59° – 60° and solidified at 57° – 58° . It was dried in the chloroform bath for analysis.

0.1120 gram substance gave 0.3096 gram CO_2 and 0.1281 gram H₂O

	Calculated for $C_{23}H_{46}O_2 \cdot CH_3$	Found
C	75.65	75.39
H	12.71	12.80

⁵ Marie, *Ann. de chim. et de phys.*, (7), vii, p. 228, 1896, *Bull. de la soc. chim.*, xv, p. 577, 1896.

⁶ *Zeitschr. f. physiol. Chem.*, xlv, p. 367, 1905.

Acetyl cerebronic acid

Thierfelder⁷ attempted to prepare acetyl cerebronic acid by the action of acetyl chloride upon cerebronic acid, he was not able to isolate the free acid in a crystalline condition, but analyzed it as the sodium salt. By the use of acetic anhydride the reaction is smooth and the product easy of isolation.

Two grams of cerebronic acid and 30 cc of acetic anhydride were heated under a reflux for two hours. Upon cooling the solution the acetylated acid separated out in a crystalline condition. This was filtered in the ice box, washed with cold alcohol several times to remove the acetic anhydride and twice recrystallized from petroleum ether. It is easily soluble in nearly all organic solvents. Acetyl cerebronic acid is a white crystalline solid, which melts at 555°-56° and solidifies at 53°-54°. Since the inactive acid was used, this product is the inactive form. The active form would melt higher.

ANALYSIS 0.1144 gram of the substance gave 0.3090 gram CO₂ and 0.1240 gram H₂O

	Calculated for C ₂₇ H ₄₂ O ₄	Found
C	73.57	73.50
H	11.90	12.15

MOLECULAR WEIGHT ESTIMATION 0.2776 gram of the substance, dissolved in pure methyl alcohol and benzene, required 6.3 cc of $\frac{N}{10}$ NaOH for neutralization.

	Calculated for C ₂₇ H ₄₂ O ₄	Found
M W	440	441

ACETYL DETERMINATION 0.0908 gram of the dried substance was dissolved in 10 cc of sodium methylate and 50 cc of pure methyl alcohol, equal to 113.6 cc of $\frac{N}{10}$ HCl, and heated under the reflux for an hour. The reaction product then required 109.8 cc of $\frac{N}{10}$ HCl for neutralization. Thus, 3.8 cc of $\frac{N}{10}$ NaOH were required in the determination. The amount calculated for the splitting off of one acetyl group and the neutralization of the cerebronic acid formed in the reaction is 4.01 cc of $\frac{N}{10}$ NaOH.

	Calculated for C ₂₆ H ₄₀ O ₃ , COCH ₃	Found
COCH ₃	9.76	9.27

⁷ Zeitschr f physiol Chem, xliii, p 27, 1904-05

Oxidation of cerebronic acid

This was carried out according to the directions of Levene and Jacobs. The action takes about two hours on the steam bath, this is better than heating with a free flame, in that bumping and possible foaming are avoided. After the addition of sodium bisulphite, the mixture is acidified, the acids filtered off, dissolved in alcohol and changed into the sodium salts. The salts from 9 grams of cerebronic acid are extracted with a liter of boiling methyl alcohol. The residue is principally the salt of the unchanged cerebronic acid. The acid obtained from the filtrate is transformed into the lithium salt and thus extracted with hot alcohol as long as a noticeable precipitate forms upon cooling the filtrate. Usually 1 liter is sufficient. The residue is the lithium salt of the new acid. This is converted into the free acid and purified through the lead salt as described above for cerebronic acid. It melts at 80° – 81° . The possible identity of this acid with lignoceric acid has already been mentioned. This will be discussed in a future paper.

0.1196 gram substance gave 0.3428 gram CO_2 and 0.1416 gram H_2O

0.5946 gram of the acid, dissolved in methyl alcohol and benzene, required 15.95 cc of $\frac{N}{10}$ NaOH for neutralization

	Calculated for $\text{C}_{21}\text{H}_{38}\text{O}_2$	Found
C	78.20	78.15
H	13.20	13.15
M. W.	368	373

Sodium salt SOLUBILITIES 100 cc methyl alcohol contains, at its boiling point, 2.186 grams, at 0° , 0.243 gram

100 cc ethyl alcohol contains, at its boiling point, 1.870 grams, at 0° , 0.080 gram

ANALYSIS 0.1004 gram of the substance gave 0.2702 gram CO_2 and 0.1099 gram H_2O

	Calculated for $\text{C}_{21}\text{H}_{37}\text{O}_2\text{Na}$	Found
C	73.77	73.40
H	12.13	12.25

Lithium salt This salt begins to soften at 172° and melts at 173 – 178° (not sharp)

SOLUBILITIES 100 cc boiling ethyl alcohol contains 0.244 gram, at 0° , 0.06 gram. 100 cc boiling methyl alcohol contains 0.210 grams and at 0° , 0.095 gram

ANALYSIS 0.1265 gram of the substance gave 0.3576 gram CO_2 and 0.1405 gram H_2O

	Calculated for $\text{C}_{25}\text{H}_{48}\text{O}_7\text{Li}$	Found
C	76.92	77.10
H	12.65	12.40

Reduction of cerebronic acid

Levene and Jacobs obtained a hydrocarbon which had nearly the required melting point for pentacosan. We have repeated the reduction under slightly different conditions and have obtained a hydrocarbon which shows the melting point given for pentacosan.

Lots of 5 grams of cerebronic acid, 25 cc of hydriodic acid of specific gravity 1.96 and 2 grams of red phosphorus were heated in sealed tubes at 125° – 130° for eight hours. The solid contents of the tubes were filtered off, after diluting the acid with much water. In the first experiments the product was crystallized from alcohol, and then changed into the sodium salt by dissolving in absolute alcohol, neutralizing to phenolphthalein with sodium methylate and evaporating to dryness. The distillation of the sodium salt was accompanied with foaming, however, and so in the later experiments the product once crystallized out of alcohol was thoroughly dried in vacuum and distilled in the vacuum of a Geryke pump. The reaction was accompanied by decomposition and some foaming, the temperature was about 320° . The distillate was taken up in absolute alcohol, neutralized with sodium methylate, evaporated to dryness, and the dry mixture extracted with ether. The residue was changed into the free acid, again distilled and treated as above. The combined ether extract was dried over anhydrous sodium sulphate, the ether removed and the product distilled in vacuum. The distillate was recrystallized from ether or alcohol. It melted at 53 – 56° , a second sample melted at 52 – 55° . Recrystallized from acetone it showed the same melting point. The product was then distilled, after the addition of a few cubic centimeters of sodium methylate solution and evaporation to dryness, the distillate recrystallized from acetone and thoroughly dried. It then melted at 53° – 54° . According to Marie⁸ pentacosan should melt at 53.5° – 54° .

⁸ Bull. de la soc. chim., xv, p. 567, 1896.

0.1140 gram of the substance gave 0.3566 gram CO_2 and 0.1444 gram H_2O

	Calculated for $\text{C}_{24}\text{H}_{48}$	Found
C	85.13	85.30
H	14.89	14.18

Reduction of $\text{C}_{24}\text{H}_{48}\text{O}_2$

The above method was then applied to other fatty acids, in the hope that it might prove a general method for the reduction of acids to hydrocarbons. The result was disappointing.⁹ In the case of $\text{C}_{24}\text{H}_{48}\text{O}_2$, obtained from the oxidation of cerebronic acid, the product of the distillation was a mixture of a large amount of the ester of the acid with a small amount of the hydrocarbon. The ester was saponified by boiling with alcoholic potash, the solution evaporated to dryness and the mixture extracted with ether. A small quantity of hydrocarbon remained. This was recrystallized from alcohol and dried in the chloroform bath. It melted at 51° – 52° . According to Krafft¹⁰ $\text{C}_{24}\text{H}_{50}$ should melt at 51.1° .

	Calculated for $\text{C}_{24}\text{H}_{50}$	Found
C	85.10	85.27
H	14.90	14.79

⁹ The reaction was also tried with stearic and palmitic acids. The product in these cases was found to be a mixture of the unchanged acid with the ethyl ester. Thus it is probably the presence of the hydroxyl group which makes the reaction possible in cerebronic acid. It is also evident that the hydroxyl group is reduced simultaneously with or after the carboxyl group, otherwise the normal acid would be obtained.

¹⁰ *Ber d deutsch chem Gesellsch*, v p 1711, 1882

CONTRIBUTIONS TO THE CHEMICAL DIFFERENTIATION OF THE CENTRAL NERVOUS SYSTEM

I A COMPARISON OF THE BRAIN OF THE ALBINO RAT AT BIRTH WITH THAT OF THE FETAL PIG

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INTRODUCTION

For the study of the progressive changes in the central nervous system during growth and senescence, the albino rat, on account of its small size, short span of life and its powers of rapid reproduction¹ is especially suited. Its growth processes are, moreover, strikingly like those of man, as has been brought out by the extensive investigations of Dr Donaldson within the past few years. It was, therefore, decided to use this animal for a study of the chemical differentiation of the central nervous system during growth.

The youngest brains which could be conveniently collected for chemical analysis were those of rats just born. As it was not certain that the rat at this period of development was sufficiently immature (chemically undifferentiated) to serve as the starting point for such a growth series, it was suggested by my brother, Dr Waldemar Koch, that the brain of the new born rat be compared chemically with the brain of the fetal pig, collected at various stages of fetal life.

By such a comparison we hoped to determine the physiological age of the rat at birth in terms of fetal pig material, and to obtain, possibly, from the pig fetus, material which would be more immature than the new born rat.

¹ H. H. Donaldson, President's Address, *Journ. of Nervous and Mental Disease*, xxxviii, p. 258, 1911.

MATERIAL AND METHODS

The rat material was obtained from the Wistar Institute of Anatomy, which supplied the brains of rats of known age, from animals which had been raised under constant conditions, two factors which are absolutely essential for such a study. The material was collected by Dr. Hatai and the method used was that adopted by the Institute. This in brief is as follows: the rat was chloroformed, the skull opened from the dorsal side, the division between the brain and the cord made at the tip of calamus scriptorius, and the brain removed. The meninges of the brain were left intact. Such blood as it contained was, therefore, included in the weight. Immediately after removal the brain was placed in a closed weighing bottle, quickly weighed to within 10 mgms.² and transferred to a wide mouthed bottle of 300 cc. capacity containing absolute alcohol. The weighing bottle was weighed back and the difference recorded as the weight of the sample. As the weight of one brain from rats at this early age is 0.2 — 0.3 gram and as it takes at least from 25 to 50 grams to make one sample for analysis, a large number of brains had to be collected (100 brains of the rat at birth for one 25-gram sample). As this covered a period of several weeks it was necessary to heat the sample from time to time in a water bath kept at a temperature of 70°C to insure a thorough penetration of the alcohol and sterilization. The amount of alcohol was so adjusted as to make the final concentration not less than 80–85 per cent. A well fitting cork stopper covered with tin-foil was now inserted and the bottle carefully shaken to insure a uniform mixture. The dates of collection of the samples were recorded, as the time a sample has been kept in some cases influences the analytical results.³ The tightly corked bottles were then shipped to the Laboratory of Biochemistry of the University of Chicago, where the samples were analyzed according to Koch's methods of tissue analysis.⁴

A coarse weighing of the brain was permissible in this instance as it was not the exact brain weight that was sought but merely data for indicating roughly when the required amount of material had been obtained.

² W. Koch, *Methods for the Quantitative Chemical Analysis of Animal Tissue*, *Journ. of the Amer. Chem. Soc.*, **xxxi**, p. 1340, 1909.

⁴ *Ibid.*, pp. 1329–64.

The fetal pig material was collected by my brother at the Chicago Stock Yards. The fetuses selected were 50, 100, and 200 mm in length. The pregnant uterus was opened and the fetuses removed. The neck-rump length of each litter was taken and if the average length corresponded to one of the three sizes mentioned above, the entire litter was taken, placed upon ice and in this chilled condition taken to the laboratory, where the brains were immediately removed, preserved and later analyzed according to the same methods used for the rat material.

RESULTS OF ANALYSES

The results from the chemical analysis of the brains from the new born rat and the adult rat are given in table I. Those of the 50, 100, and 200 mm pig fetuses are recorded in table II, and table III gives the summary of all the averages which have been taken from the figures which were most consistent. The brain of the 200 mm pig fetus was plainly more differentiated, it is therefore left out of table III and of the final discussion of results.

DISCUSSION OF CHEMICAL RESULTS

Before taking up a comparison of the new born rat with the pig fetus it may be well to state, briefly, the chief chemical changes in nervous tissue during growth. It is well known that the chemical composition of a tissue varies with age and that the water content is one of the most important variables. Donaldson states that, "the progressive diminution of the percentage of water in the brain is a function of age and is not significantly modified by any conditions to which the animals have been thus far experimentally subjected."⁵ He suggested that this "is to be regarded as an index of fundamental chemical processes, which take place in the more stable constituents of the nerve cells."⁶ The principal chemical differences due to growth, noted by my brother, are, "a decrease in moisture, proteins, extractives, and ash as the brain increases with age, and an increase in cerebroside, sulphatides,

⁵ H. H. Donaldson. On the percentage of Water in the Brain and in the Spinal Cord of the Albino Rat, *Journ. of Neurol. and Psychol.*, x, p. 143, 1910.

⁶ *Ibid*

phosphatides, and cholesterol, in other words, an increase in substances which predominate in the fibres (medullated sheath) during growth¹⁷ These same differences are to be found in all

TABLE I

Relative proportion of the proximate constituents of the brain of the albino rat at birth and when adult

	ALBINO RAT (AT BIRTH)		ALBINO RAT (ADULT)
Moist weight of one brain	0 25	0 25	1 667
Solids in per cent	10 42	10 42	21 9
Dry weight of one brain	0 026	0 026	0 380
Number of brains in sample	100	100	31

In relative proportions of solids

Proteins	57 16	57 30	48 5
Phosphatides	14 8	15 6	22 0
Cerebrosides	0 0*	0 0	9 0
Sulphatides	1 5	1 4	4 6
Organic extracts } Inorganic const }	16 5	19 3	9 8
Cholesterol } Undetermined } †	(10 04)	(6 4)	(6 1)
Total S	0 96	1 04	0 58
Total P	1 82	1 92	1 39

Distribution of sulphur in per cent of total S

Protein S	31 02	30 02	64 2
Lipoid S	3 2	2 8	15 6
Neutral S	49 14	47 26	14 2
Inorganic S	16 6	19 95	6 0

Distribution of phosphorus in per cent of total P

Protein P	13 3		6 8
Lipoid P	33 2	33 0	67 6
Water-soluble P	53 5	53 6	25 6

Mendel L Amer *Journ of Physiol* xxi p 104 1908
† By difference

¹⁷ W Koch and S A Mann A Comparison of the Chemical Composition of Three Human Brains at Different Ages, *Journ of Physiol*, xxxvi, pp 1-3 (From the Proceedings of the Physiological Society, November 23, 1907)

TABLE II

Relative proportions of the proximate constituents of the brain of the fetal pig at different ages

Year of analysis	50 MM PIG FETUS			100 MM PIG FETUS			200 MM PIG FETUS
	11	12	12	11	12	12	11
Moist weight of one brain	0 40	0 43	0 47	1 8	1 91	2 15	10 1
Solids in per cent	8 75	9 87	9 04	9 1	8 98	8 98	
Dry weight of one brain	0 035	0 042	0 042	0 164	0 171	0 193	
Number of brains in sample	65	111	109	35	27	27	

In relative proportions of solids

Proteins	56 6	58 2	54 61	51 5	51 81	52 34	43 8
Phosphatides	13 0	15 04	15 79	15 7	16 31	14 85	17 2
Cerebrosides							
Sulphatides	2 4?	0 8	1 05	1 8?	0 96	0 84	0 00?
Organic extract	22 20	20 5	23 84	24 2	24 92	25 44	23 2
Inorganic const }							
Cholesterol	2 4*	2 4*	2 4*	4 4*	4 4*	4 4*	
Undetermined†	(3 4)	(3 06)	(2 31)	(2 4)	(1 6)	(2 13)	(8 42)
Total S	0 67	0 59	0 58	0 59	0 57	0 55	0 55
Total P	1 74	1 85	1 90	1 76	1 91	1 82	1 45

Distribution of sulphur in per cent of total S

Protein S	54 3	57 3	55 8	58 4	57 8	55 66	60 9
Lipoid S	7 2?	2 6?	3 59	6 1?	3 36	2 98	0 00?
Neutral S	29 3	29 4	27 6	26 7	28 97	31 68	25 5
Inorganic S	9 0	10 6?	13 0?	9 0	9 93	9 6	13 33

Distribution of phosphorus in per cent of total P

Protein P		15 7	14 0		14 5	14 6	5 2
Lipoid P	31 6	32 4	29 6	35 5	34 5	31 6	46 3
Water-soluble	53 6	51 8	56 2		50 9	53 8	48 5

* Mendel L. *Amer Journ of Physiol* xxi p 103 1903

† By difference

(?) Indicates doubtful result

TABLE III

Relative proportions of the proximate constituents of the brain of the fetal pig at different ages compared with the brain of the albino rat at birth (Average of the foregoing determinations)

	PIG FETUS		ALBINO RAT	
	50 mm	100 mm	at birth	adult
Moist weight of one brain	0 433	1 90	0 25	1 667
Solids in per cent	9 22	8 99	10 42	21 9
Dry weight of one brain	0 039	0 171	0 026	0 380
Number of brains in sample	95	30	100	31

In relative proportions of solids

Proteins	56 47	51 88	57 23	48 5
Phosphatides	15 41	15 62	15 2	22 0
Cerebrosides*	0 0	0 0	0 0	9 0
Sulphatides	0 92	0 90	1 45	4 6
Organic extract } Inorganic const }	22 18	24 69	17 9	9 8
Cholesterol	2 4†	4 4†		
Undetermined†	(2 59)	20 49	(8 22)	(6 1)
Total S	0 585	0 57	1 00	0 58
Total P	1 83	1 83	1 87	1 39

Distribution of sulphur in per cent of total S

Protein S	55 8	57 28	30 52	64 2
Lipoid S	3 13	3 17	3 0	15 6
Neutral S	28 7	29 11	48 2	14 2
Inorganic S	9 83	9 51	18 27	6 0

Distribution of phosphorus in per cent of total

Protein P	14 8	14 55	13 3	6 8
Lipoid P	31 2	33 8	33 1	67 6
Water-soluble P	53 8	52 3	53 55	25 6

* Cerebrosides not determined in fetal brains Not present according to Mendel

† Mendel L Amer Journ of Physiol xxi, p 103 1903

‡ By difference

nervous tissue during growth and may therefore be used in making a comparison between the brain of the new born rat and that of the fetal pig to determine which is the more immature

'We may now proceed to consider in detail the comparison of the various constituents⁸ in the brain of the new born rat and the pig fetus

Water The per cent of water in the brain of the new born rat is closely similar to, but a little lower than, that of either the 50 mm or 100 mm pig fetus This would indicate that the rat is of about the same physiological age as these fetuses, since the differences are within the limits of error

Protein The per cent of protein in the total solids is higher in the brain of the new born rat than in either that of the 50 or the 100 mm pig fetuses Since the per cent of protein is highest in the youngest material, this is an indication that the rat's brain is less mature than that of the 100 mm pig fetus, but not very different from the 50 mm fetus

Phosphatides The per cent of phosphatides is the same in the new born rat as it is in the 50 and the 100 mm pig fetus This would indicate a close agreement in physiological age between these two This is the lowest phosphatide content yet obtained in an analysis of the brain tissue and approaches that observed in the suprarenal, which, among all the organs, comes closest to that of nervous tissue in chemical composition

Cerebrosides These are absent in both the new born rat, and in the pig fetus, as is to be expected in nervous tissue before myelination

Sulphatides The percentage of sulphatides is about the same in the new born rat as in the pig fetus, which indicates the same age

Organic extractives and inorganic constituents These are somewhat higher in the pig fetus than in the new born rat and, except as this is associated with the greater per cent of lymph in the embryonic material, it would indicate it to be more immature than the new born rat

⁸ The nature and significance of the constituents will be discussed in the third paper of this series

Cholesterol The figures for cholesterol were not determined by me, but were taken from Mendel⁹ and incorporated here for the sake of completeness

This leaves *undetermined* from 2 to 3 per cent which is not more than would be expected in the errors involved in making so many determinations from one tissue and calculating approximate constituents from assumed factors

The distribution of sulphur in per cent of total sulphur is widely different in the two forms, but as this is not correlated with age but is apparently a species peculiarity, the results are not out of harmony with the foregoing conclusions

The distribution of phosphorus between the protein, lipid and water-soluble phosphorus is closely similar in the rat and the 50 and 100 mm pig fetus, showing the physiological ages to correspond

The remarkably high figure for neutral and inorganic sulphur in the rat at birth requires an explanation but it is not possible to give this with the data so far at hand

The general conclusions from these figures are, that from a chemical standpoint the brain of the new born rat is about as immature as that of the 100 mm pig fetus, being on the whole a little less differentiated than the latter

The differences between the brain of the 50 mm and the 100 mm pig fetus are not marked, and this would indicate that there occurs between these ages an increase in weight unaccompanied by any significant change in chemical composition This would correspond with the results of Mendel¹⁰ and Raske¹¹ who found that in the brains from these young fetuses there is no chemical distinction between grey and white matter Moreover the brain of the 50 mm pig fetus is the youngest which it is practicable to obtain for analysis and even at this age the tissues are so watery and filled with lymph that some error is thereby introduced in the analysis of the constituent tissues

Since the brain of the 50 mm pig fetus shows no material differences from that of the 100 mm pig fetus and the latter is no more

⁹ L B Mendel and Charles S Leavenworth *Chemical Studies on Growth*
IX. Notes on the Composition of Embryonic Muscular and Nervous Tissues
Amer Journ of Physiol, xxi, p 103, 1908

¹⁰ *Ibid*

¹¹ Raske *Zeitschr f physiol Chem*, x, p 340, 1886

immature chemically than that of the new born rat, it appears that the new born rat's brain is as young nervous material as can conveniently be analyzed at present and it forms, therefore, a convenient starting point for the study of the chemical differentiation of the central nervous system during growth

CHEMICAL RESULTS CONFIRMED BY PHYSIOLOGICAL AND ANATOMICAL DATA

It is astonishing that chemically the brain of the new born rat should be as immature as that of the 100 mm pig fetus, but, surprising as this fact is, it is substantiated by a comparison of the structure of the cerebellum of these two animals and of their behavior at the time of birth

It is a well-known fact that the rat is born in a very immature state, with its eyes shut, and when first born, is capable only of movements involved in sucking, bending the body and tail and making a squeaking noise¹² The pig, on the other hand, "is born with its eyes open and requires no assistance as a rule in making its start in life It is more or less able to walk around as soon as born"¹³ Such a state of activity in the rat is not reached until the period of weaning 17-21 days after birth

This difference in physiological behavior is correlated with the relative development of the cerebellum of the two animals, particularly as indicated by the development and transformation of the outer granular layer of cells A comparison of this layer in both animals, founded on the observations of Addison¹⁴ who studied the different layers of the cerebellum in the albino rat, and of Takasu¹⁵ who studied these same layers in the pig fetus, brought out the following facts

¹² Wm H F Addison The Development of the Purkinje Cells and the Cortical Layers in the Cerebellum of the Albino Rat, *Journ of Comp Neurol*, xvi, p 476, 1911

¹³ Forbes personal communication

¹⁴ Wm H F Addison *Journ of Comp Neurol*, xvi, p 464, 1911

¹⁵ K Takasu Zur Entwicklung der Ganglienzellen der Kleinhirnrinde des Schweines, *Anat Anz*, xvi, pp 225-32, 1905

	RAT	PIG
First appearance of cells in outer granular layer	19-day fetus	50 mm fetus
Division of layer into two zones inner and outer	At birth	100 mm fetus
Disappearance of cells from inner zone of layer	21 days after birth	300 mm fetus

The first appearance of the cells in the outer granular layer of the cerebellum in the rat is in the 19-day fetus, and in the pig in the 50 mm pig fetus. At this time the cells are settled in a thin layer (two rows deep) around the outer edge of the cerebellar cortex. This layer increases until a considerable depth is filled in with cells which soon separate into two strata, an outer and an inner, this separation takes place in the rat at birth, and in the pig fetus when it is 100 mm in length. The cells from the outer granular layer now begin to migrate to the inner granular layer and the disappearance of the cells from this outer granular layer, which corresponds with the time of securing motor control in an animal, occurs in the rat at the twenty-first day of life, and in the pig when this is from 200 to 300 mm in length, or at birth. These facts show, therefore, that the new born rat is as developed with respect to motor activity as the 100 mm pig fetus, and that the rat at weaning (17-21 days after birth) and the pig at birth are at corresponding physiological ages. The conclusion from this anatomical comparison, namely, that the 100 mm pig fetus and the rat at birth are of like physiological age, fully confirms, therefore, the conclusion drawn from both chemical and physiological evidence already adduced.

We now ask the question, how far this result, that the nervous system of the new-born rat is chemically as old as that of the 100 mm pig fetus, agrees with observations made by Donaldson, that the rate of growth and percentage of water in the mammalian nervous system (represented by the brains of man and the rat) agree in the two forms at equivalent ages, thus indicating that the nervous systems are in corresponding physiological states at equal fractions of the life cycles.¹⁶

¹⁶ H. H. Donaldson. A Comparison of the White Rat with Man in respect to the Growth of the Entire Body, *Boac Anniversary Volume*, 1906, pp 5-26

It remains therefore to inquire whether the chemical and behavior relations between the rat and pig which have just been pointed out, occur at equivalent ages in these two forms

Great difficulty was experienced in finding any statements concerning the age of the pig fetuses. The statements of different authors did not always agree, but the two which agreed closest were those of Bradley¹⁷ and Coe¹⁸. Bradley compared the length of the embryos with the time from coition, Coe estimated the age from the rate of development of embryos of other mammals. While considerable uncertainty thus attaches itself to these figures¹⁹ it may be assumed that the 50 mm pig fetus is about 40 days old from conception, the 100 mm fetus is 55-62 days, and the 200 mm fetus is from 88-90 days from conception.

We find in the rat the period of gestation is 21 days and its span of life three years (Donaldson), or a total age of 1116 days, in the pig the period of gestation is 125 days and its normal span of life, as far as could be ascertained, is 20 years,²⁰ or 7425 days. The rat, therefore, lives about one-sixth as long as the pig. Assuming that the rat at birth has lived $\frac{21}{1116}$, or $\frac{1}{53}$, of its total life, the 60-day pig fetus will have lived $\frac{60}{7425}$, or $\frac{1}{123}$, of its life. It appears then, if the total length of life given is correct for both animals and the numbers used for the divisors in each case are really comparable as they stand, that we do not have corresponding physiological conditions of the brain at equivalent ages, for these brains

¹⁷ O C Bradley On the Development of the Hind Brain of the Pig, *Journ of Anat and Physiol*, 21, Part I, p 1

¹⁸ Mendel refers to Professor Coe in Chemical Studies on Growth. I The Inverting Enzymes of the Alimentary Tract, especially in the Embryo, *Amer Journ of Physiol*, xx, p 90, 1907-1908

¹⁹ Bradley makes the statement, that "although the age of the different embryos is given, it is not intended that it should signify more than the time which elapsed between the time of coition and the time when the mother was destroyed.

In embryos taken from two litters it not infrequently happens that those which should be further advanced in development, judging from the period which has elapsed since sexual congress took place, are as backward, or even more backward, than those of the 'younger' litter." A more definite way to determine the age of an embryo would be, according to Mall, by ossification. No data were available however for a comparison between the rat and the pig.

²⁰ Longevity, *Encyclopædia Britannica* (Eleventh Edition), xvi p 975

are found to be in corresponding states at the $\frac{1}{18}$ and the $\frac{1}{18}$ part of the total life cycles. Had the relation, in the form stated above, held, these fractions should have been identical. It is only fair to add, however, that in view of the absence of precise information concerning the pig and in view of the fact that the early days of gestation are used for cell division accompanied by only slight differentiation, too much stress should not be laid on the relation here given.

On the other hand, instead of taking the end of life as the fixed point of our calculations, we may consider the time when motor control is obtained to indicate closely corresponding states of the central nervous system. In the rat, motor control is obtained at 42 days from conception, and in the pig at 125 days, that is, at birth. If the law of corresponding states is correct, the nervous system of these two animals should be in corresponding conditions at the same fractions, either $\frac{1}{3}$, $\frac{2}{3}$, or $\frac{3}{4}$ of these periods. This is found to be the case, for the rat is born after 21 days' gestation. This would be just half way between the two fixed points of conception and time of gaining motor control and this corresponds in the pig to just one-half of its gestation period or about 62 days, which is the age of the 100 mm pig.

It was actually found, both chemically and anatomically, that the nervous systems of these two animals were in the same state of development at these respective periods and it appears from these observations that Donaldson's law may hold, if put in the form the nervous systems of mammals are in the same physiological state at equal fractions of their total periods of development.

In conclusion it gives me great pleasure to thank Dr H H Donaldson and Prof A P Mathews for their many suggestions in connection with this problem and for their aid in getting this paper ready for publication. The problem itself was suggested to me by my brother and forms the first of a series of papers which are to follow from time to time, on the chemical differentiation of the central nervous system, and on which he was engaged at the time of his death. The work was carried out in the Laboratory of Biochemistry and Pharmacology of the University of Chicago and was aided by a grant from the Wistar Institute of Anatomy and Biology, Philadelphia.

SUMMARY

1 A quantitative determination of the constituents of the brain of the albino rat at birth shows it to be chemically as undifferentiated as the brain of a 50 mm or 100 mm pig fetus

2 There is little difference in chemical composition between the 50 mm and the 100 mm pig fetus brain

3 Since the 50 mm fetus brain is the youngest which can be analyzed and this closely resembles the 100 mm fetus, and this in turn is no more immature than the new born rat, it appears that the brain of the new born rat is sufficiently immature to serve as a starting point in a study of the chemical differentiation of the brain during growth

4 That the brain of the new born rat is as immature as the 100 mm pig embryo is shown, also, by the similarity of the changes in the outer layer of cells of the cerebellar cortex in both animals previous to gaining motor control, and by the animal's behavior at this period

5 If the nervous systems are assumed to be in corresponding states when motor control is obtained, and Donaldson's law is correct, that the nervous system is in the same state at corresponding physiological ages, then the brain of the rat at birth should correspond chemically with the 100 mm pig fetus brain. This is found to be the case

CONTRIBUTIONS TO THE CHEMICAL DIFFERENTIATION OF THE CENTRAL NERVOUS SYSTEM

II A COMPARISON OF TWO METHODS OF PRESERVING NERVE TISSUE FOR SUBSEQUENT CHEMICAL EXAMINATION

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(Received for publication, February 20, 1913)

With valuable biological material it is sometimes desirable to make water estimations and the estimations of the other constituents on the same sample. This can be done somewhat indirectly if the method already described¹ of placing the fresh, weighed tissues immediately in 95 per cent alcohol is used.

To see whether tissues in which the water had been determined by drying could thereafter be analyzed by the methods referred to above and would yield the same proportion of the various constituents as these same tissues treated by the alcohol method, an analysis was made of the brains and spinal cords of albino rats which had been dehydrated in these two ways. The possible drawbacks of the heat method of determining moisture, namely, the oxidation or decomposition of part of the material and the evaporation of volatile constituents, are obvious, but we had no definite knowledge of how serious the errors involved in the method might be in practice.

To determine to what extent these changes took place and what they were, we analyzed material which had been dried at 95°C for one week and which at the end of this time had been placed in alcohol, and compared it with similar material which had been placed directly in alcohol. The results are given in the table.

It may be seen by a comparison of the results of the two analyses in the table, that decompositions seriously affecting the analyses are produced by heat drying, particularly in the case of the brain. The differences are most marked in the phosphorus compounds.

¹ Koch, W. *Journ Amer Chem Soc*, 22, pp 1353-4, 1909

Comparison of brains and cords dried at 95°C with brains and cords placed directly in alcohol without heating

	ENCEPHALON		CORDS	
	Direct into alcohol	Dried at 95 C	Direct into alcohol	Dried at 95 C.
Laboratory number	W 13	W 18	W 9	W 20

In per cent of total solids

Proteins	48 5	47 9	32 8	29 6
Phosphatides	22 0	16 2	25 3	22 1
Cerebrosides	8 4	(?)	12 5	14 4
Sulphatides	4 5	4 6	7 0	6 7
Organic ext	9 8	12 4	7 6	8 0
Inorganic const				
Undetermined lipoids	6 8*		14 8*	19 2*
Total S	0 58	0 59	0 45	0 42
Total P	1 39	1 31	1 44	1 42

Distribution of sulphur in per cent of total S

Protein S	63 8	64 6	53 7	53 3
Lipoid S	15 6	15 6	30 9	32 1
Neutral S	14 5	14 0	10 3	9 5
Inorganic S	6 1	6 0	5 0	5 0

Distribution of phosphorus in per cent of total P

Protein P	6 8	8 1	5 6	5 0
Lipoid P	67 6	55 1	77 4	69 2
Water Sol P	25 6	36 8	17 0	25 8

* Obtained by difference

By drying there has been a destruction of the phosphatides involving a change in the distribution of phosphorus in per cent of total phosphorus, that is, a considerable amount of lipid phosphorus is changed to water-soluble phosphorus. There was no change in the sulphur distribution. It will be noticed that the phosphatides of the cord are more resistant to heat than those of the brain, a point of sufficient interest to justify repetition.

We conclude, then, that the determination of water by drying at 95°C cannot safely be used, if it is desired to determine in the same sample the relative proportions of the solid constituents, and that the indirect method already described is far superior for this purpose.

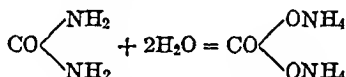
A RAPID CLINICAL METHOD FOR THE ESTIMATION OF UREA IN URINE

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As is well known, the conversion of urea into ammonium carbonate can be brought about by an enzyme found in numerous bacteria,¹ fungi² and higher plants³



An enzyme solution exerting this activity is easily and conveniently prepared from the soy bean (*Glycine hispida*)⁴ By extracting the ground tissue with acidified water and filtering, a perfectly clear solution is obtained which under proper precautions can be preserved for a considerable length of time. A solution in which urea is to be determined is treated with a portion of this extract and, after the conversion into ammonium carbonate is complete, the alkalinity of the solution is determined by titration with standard acid using an indicator which is not appreciably sensitive to

¹ Musculus *Compt rend de l'Acad des Sci*, lxxxii, p 334, 1876, Leube *Virchow's Archiv*, c, p 540, 1885, Lea *Journ of Physiol*, vi, p 136, 1886, Miquel *Bull de la soc chim de Paris*, xxix, p 387, xxxi, p 391, xxxii, p 136, also, *Compt rend de l'Acad des Sci*, cxi, pp 397, 501

² Shibata *Beitr z chem Physiol u Path*, v, p 384, 1904

³ Takeuchi *Journal of the College of Agriculture, Tokyo*, i, p 1, 1909, Keisel *Zeitschr f physiol Chem*, lxxv, p 169, 1911, Zemplen *Zeitschr f physiol Chem*, lxxix, p 229, 1912, *Zeitschr f angew Chem*, xxv, p 1560, 1912

⁴ This enzyme was discovered in the seeds and seedlings of the soy beans by Takeuchi who employed its hydrolyzing power for the manufacture of ammonium sulphate from urine *loc cit* and also *Chem Zeitung*, xxv, p 408, 1911

carbonic acid From the amount of standard acid used, the amount of urea originally present can be ascertained by an obvious calculation

With solutions of pure urea the figures obtained are practically theoretical and, even when applied to urine itself, the results are all that could be desired in a clinical method, the divergence from the theoretical being about 2 per cent

The procedure to be described is peculiarly useful in its application to pathological urines since the presence of glucose and protein, usually so annoying in the estimation of urea, are without influence here and need not even be removed

A large number of methods based upon different principles have been devised for the estimation of urea in urine For scientific investigations, the one proposed by Folin⁵ in its various modifications, the Morner-Sjoqvist⁶ method and its combination with Folin's for urines containing sugar, and finally the procedures devised by Benedict⁷ have been extensively used All of these methods, however, necessitate an independent determination of the preformed ammonia in the urine, and in general are not sufficiently simple to find favor for rapid clinical work The various methods, based upon the decomposition of urea by an alkaline solution of sodium hypobromite and a measurement of the nitrogen evolved, are, therefore, still used for clinical purposes As is well known, however, they are very inaccurate and the results are vitiated by the presence of ammonium salts

Miquel⁸ in his investigations on the various urea-fermenting bacteria suggests that filtered cultures of *Micrococcus ureae* could be utilized to convert the urea of the urine into ammonium carbonate and to estimate its amount However, he cites but one experiment in this connection Hudson⁹ has proposed a very accurate method for the determination of cane sugar by means of

⁵ Folin *Zeitschr f physiol Chem*, **xxii**, p 504, 1901, **xxvi**, p 333, 1902, **xxvii**, p 548, 1902-03, *Amer Journ of Physiol*, **xiii**, p 45, also, this *Journal*, **xi**, p 45, 1912

⁶ Morner *Skand Arch f Physiol*, **xv**, p 297, 1903

⁷ Benedict and Gephart *Journ of Amer Chem Soc*, **xxv**, p 1760, 1903, Benedict this *Journal*, **viii**, p 405, 1910-11

⁸ *Compt rend de l'Acad des Sci*, **cxi**, p 501, 1890

⁹ *Journ of Ind and Eng Chem*, **ii**, p 143, 1910

the invertase of yeast. The enzyme solution employed hydrolyzes cane sugar completely, while it is without action upon maltose, lactose, starch, dextrins, pentosans, and the natural glucosides. The use of the fermentation of glucose brought about by yeast has been employed clinically for the determination of sugar in pathological urines.

EXPERIMENTAL

Preparation of the enzyme solution. The soy beans are ground to a fine powder which can be preserved in well-stoppered dry bottles for months without appreciable loss of activity. Twenty-five grams of this powder are mixed with 250 cc of distilled water, and allowed to stand with occasional agitation for about an hour. Twenty-five cc of $\frac{N}{10}$ hydrochloric acid are now added, and the mixture allowed to remain a few minutes longer (best in a water bath at about 35°), when a large proportion of the protein of the bean extract is precipitated. The mixture is filtered, the filtrate treated with a few drops of toluene and preserved for use in a stoppered vessel. On standing the originally clear fluid becomes opalescent, and finally a precipitate is formed, but the solution remains sufficiently active for use in the method at least five days after its preparation when kept at the room temperature¹⁰. This solution is alkaline to methyl orange, and 2 cc generally require from 0.28 to 0.34 cc of $\frac{N}{10}$ hydrochloric acid for neutralization. This factor should be determined once for 2 cc of each preparation and can then be employed as a correction as long as the solution is used. The alkalinity is apparently constant from day to day. If for any reason the extract should not be distinctly alkaline to methyl orange, less acid should be used in its preparation, as an extract which reacts acid to methyl orange is scarcely active.

Execution of the method. Two 5 cc portions of the urine are measured into flasks of 200–300 cc capacity and diluted with distilled water to about 100–125 cc. Two cc of enzyme solution are added to one flask, a few drops of toluene to each and the solution allowed to remain, well stoppered, at room temperature over-

¹⁰ The enzyme solution loses its activity much more rapidly at a higher temperature (35°), and would, therefore, probably keep better in an ice chest. I am at present investigating the problem of preparing a purer solution and finding means of preserving its activity.

night The fluid in each flask is titrated to a distinct pink color with $\frac{N}{10}$ hydrochloric acid, using methyl orange as an indicator The amount of hydrochloric acid required for the contents of the flask containing the urine and enzyme solution less the amount used for 5 cc of urine alone and that previously determined for 2 cc of enzyme solution, corresponds to the urea originally present in the sample of urine Since 1 cc of $\frac{N}{10}$ hydrochloric acid is equivalent to 3 mgm of urea, the number of cubic centimeters required multiplied by 0.6 gives the value of urea expressed in grams per liter of urine The completeness of the conversion of the urea into ammonium carbonate can be tested at any time by performing one determination as described and a duplicate which is allowed to stand at 35°-38° The results of the two experiments should be identical provided the hydrolysis is complete, but if the enzyme has not converted all the urea in the first determination, the second will give higher results The same purpose is attained, of course, by allowing the second determination to proceed at the same temperature but some hours longer than the first

Length of time necessary for the complete conversion The time required for complete hydrolysis of the urea depends upon the quantity of urine used, the concentration of the urea, the amount of enzyme present, and the temperature of action The velocity of the reaction is approximately twice as rapid at 35° as at 25°, and directly proportional to the enzyme concentration within certain limits As shown by the following data, 5 cc of urine or 5 cc of a 2 per cent urea solution required about 3 hours for complete hydrolysis at a temperature of 35°

TIME	2 PER CENT UREA SOLUTION CORRECTED VALUE CC 0.1 N HCL	URINE CORRECTED VALUE CC 0.1 N HCL
hours		
1	23.45	26.03
2	32.55	37.00
3	33.15	40.74
4		40.78
24	33.10	

The conversion is complete in less than 1 hour at 35°, when 10 cc of the enzyme solution are employed instead of 2 cc

TIME	CORRECTED VALUE cc 0.1 N HCL
<i>minutes</i>	
45	33 26
80	32 68

A cloudiness, however, is produced on titrating a solution containing 10 cc of the enzyme mixture, which renders the end point uncertain and the procedure less accurate. With the use of only 2 cc of enzyme solution this cloudiness is scarcely noticeable. If more rapidity is required than is attained by the method as outlined, digestion for three hours at a temperature of 35°–38° will suffice, or, if accuracy is to be sacrificed to rapidity, less urine and more enzyme solution can be employed.

Application of the method to pure urea solutions The following table explains itself

GRAMS UREA 100 CC USED	CC 0.1 N HCL REQUIRED	CORRECTION FOR ENZYME SOLUTION	GRAMS UREA PER 100 CC FOUND
0.500	8.55	0.28	0.4962
1.000	16.79	0.28	0.9906
2.000	33.50	0.30	1.9920
2.000	33.45	0.30	1.9890

Application of the method to urine The following results obtained with a sample of normal urine are a fair example of the agreement by this method. Two duplicate determinations carried out at room temperature gave 2.190 and 2.187 grams of urea per 100 cc. Two similar determinations on the same sample but carried out at 37° gave 2.200 and 2.173 grams per 100 cc. The effect of adding a known amount of urea to the urine is shown below. Two cc portions of urine were used and 5 or 10 cc portions of a 1 per cent urea solution.

AMOUNT UREA ADDED EXPRESSED IN CC 0.1 N HCL	REQUIRED BY URINE ALONE CC 0.1 N HCL	REQUIRED BY URINE AND UREA CC 0.1 N HCL	DIFFERENCE UREA FOUND EXPRESSED IN CC 0.1 N HCL
16.67	15.35	31.86	16.51
16.67	16.52	33.00	16.48
33.33	15.35	48.55	33.20

The method was applied to solutions of composition stated below. These solutions contained approximately the amount of urea normally present in the urine.

	SOLUTION I GRAMS PER LITER	SOLUTION II GRAMS PER LITER	SOLUTION III GRAMS PER LITER	SOLUTION IV GRAMS PER LITER
Urea	20 00	10 00	20 00	20 00
Creatinine	0 72	0 36		
Hippuric acid	0 50	0 25	0 50	
Uric acid			0 50	
NaCl	10 00	5 00	10 00	10 00
KH ₂ PO ₄	1 40	0 70	1 40	1 40
Na ₂ HPO ₄	1 40	0 70	1 40	1 40
K SO ₄			1 50	1 50
MgSO ₄			0 25	0 25
NH ₃			0 50	

Solution I contained also potassium sulphate, potassium acetate and acetic acid, as the creatinine solution was prepared from creatinine zinc alum by precipitating the zinc with hydrogen sulphide in the presence of potassium acetate.

Solutions I, II and IV were acid to litmus and alkaline to methy orange, while solution III was alkaline to both litmus and methy orange, the former being the condition of acidity in normal urine.

SOLUTION NO	CORRECTED VALUE CC 0.1 N HCL FOUND	THEORETICAL VALUE CC 0.1 N HCL CALCULATED
I	32 85	33 33
I	32 75	33 33
I	32 89	33 33
II	16 42	16 67
II	16 45	16 67
II	16 31	16 67
III	32 98	33 33
III	32 86	33 33
III	32 86	33 33
III	32 81	33 33
IV	32 94	33 33
IV	32 97	33 33
IV	32 89	33 33
IV (boiled)	33 26	33 33
IV (boiled)	33 21	33 33

The uric acid and hippuric acid were converted into the sodium salts by the addition of the theoretical quantity of $\frac{N}{10}$ sodium hydroxide

The last two samples were treated with an excess of $\frac{N}{10}$ hydrochloric acid, boiled to expel the carbon dioxide, cooled, and titrated with alkali

Application of the method to urine containing sugar and albumin
Variable quantities of egg albumin and glucose were added to a sample of normal urine, and the urea determined

ADDED TO 5 CC URINE	5 CC URINE ALONE REQUIRED CC 0.1 N HCL	5 CC URINE AFTER HYDROLYSIS RE- QUIRED CC 0.1 N HCL	CORRECTED VALUE CC 0.1 N HCL
	1 85	40 66	38 51
	1 91	40 80	38 59
0.2 cc egg-albumin	2 12	40 85	38 43
1.0 cc egg-albumin	2 80	41 48	38 38
0.1 gram glucose	1 90	40 65	38 45
0.1 gram glucose	1 90	40 71	38 57
0.5 gram glucose	1 87	40 74	38 57
0.5 gram glucose	1 87	40 81	38 64

Effect of the carbon dioxide on the titrations The results obtained on pure urea solutions, urea solutions added to urine, and urea in the presence of various nitrogenous substances and salts are consistently 1 to 2 per cent lower than the theoretical values. In the two experiments in which the carbon dioxide was removed by boiling, the figures differ from the theoretical by only a few tenths of one per cent. The lower values are undoubtedly caused by the effect of the carbonic acid on the indicator in the presence of neutral salts, especially the ammonium chloride formed during the titration.¹¹ The magnitude of this error was determined by titrating a solution of ammonium carbonate with and without the removal of the carbon dioxide.

¹¹ This is, of course a well-known fact. However, the extent of the error involved by using methyl orange with carbon dioxide in the presence of neutral salts is generally underestimated. Acree and Brunell have already pointed out this fact (see *Amer Chem Journ*, XLVI, p 120, 1906). The fact that the reaction may be slightly reversible must not be overlooked in this connection, although no evidence of such reversibility has so far been obtained.

Ten cc of ammonium carbonate solution required 29.78 and 29.81 cc of $\frac{N}{16}$ HCl. Removing the carbon dioxide, 10 cc required 30.03 and 30.05 cc of $\frac{N}{16}$ HCl. The error is, therefore, about 0.8 per cent. Removing the carbon dioxide would, of course, increase the accuracy of the method, but as the control with 5 cc of urine alone cannot be subjected to the same treatment (adding excess acid, boiling, and retitrating) on account of an hydrolysis of the urea, it is not introduced as an essential feature. The carbon dioxide could also be removed by passing a rapid current of air through the acidified solution, or pumping out *in vacuo*.

The use of this refinement, however, is scarcely desirable when dealing with urine since results within an error of 2 per cent¹² can be secured by the more rapid procedure.

¹² The method described in this paper is recommended for the rapidity with which it can be executed and the very fair degree of accuracy secured. But by the introduction of certain refinements (the distillation of ammonia before the titration is made) the method can be made sufficiently accurate for scientific work such as the determination of urea in the blood. I am at present engaged with problems of this nature.

THE LIPINS OF THE HEART MUSCLE OF THE OX¹

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(Received for publication, February 21, 1913)

The question of how lipins are combined in cellular material is of great importance. The writer in another connection³ has reviewed our knowledge of all the known combinations in which lipins may exist in cellular material.

On account of the labile nature of these substances it is possible that our methods of extraction may change the true nature of these substances. Recently MacLean and Williams,⁴ by extracting dog's liver in the cold with ether and alcohol, show that as much as 84 per cent of the total extract is of the nature of phosphatide (phospholipin) and conclude "that the essential fat of the liver, and probably of certain other organs is really phosphatide, and under certain circumstances, if care be taken to avoid disintegration during the process of extraction, it may be practically the only one found in any appreciable amount in the combined part of the fat."

In light of the recent observations of Leathes⁵ and his co-workers, which show the liver to be an active agent in the elaboration of

¹ A preliminary account of this work was published in *Science*, xxvii, p 221, 1911, *Biochem Bull*, i, p 114, 1911.

Most of the analytical data presented in this paper were obtained while working in the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York City.

² *Studies in Cancer and Allied Subjects, Conducted under the Auspices of the George Crocker Special Research Fund*, Volume III, Department of Biological Chemistry.

³ *Biochem Journ*, iv, p 455, 1909.

⁴ Mottram *Journ of Physiol*, xxviii, p 281, 1909, Leathes *Arch f exp Path u Pharm (Schmiedeberg's Festschrift)*, 1908, p 327, *Lancet*, clxxvi, p 594, 1909, Hartley *The Fats*, 1910, *Journ of Physiol*, xxvi, p 17, 1907, *ibid*, xxviii, p 353, 1909, Hartley and Mavrogordato *Journ of Path and*

the complex "fats" and that it effects the desaturation of fatty acids, it is possible that this organ might contain a larger amount of phospholipin than other organs supposedly not so important in the elaboration of substances of the nature of the complex fats, and that it was not so much a question of the disintegration of the phospholipins during extraction as it was whether the liver "fat" might be different from the fat of other organs

Paton⁶ however obtained the following results as regards the amount of lecithin in the ether extract of liver of rabbits. A rabbit liver was divided into two parts, one part being dried at 38°C for forty-eight hours, the other dried for eight hours on a water bath. The liver dried at 38° yielded 56.1 per cent of lecithin in the ether extract and the liver dried on the water bath gave 58.4 per cent of lecithin in the ether extract, showing that very little change had taken place.

The following experiments show that *only about 40 per cent of the ether and alcohol extract of heart muscle of the ox is composed of phospholipin and practically no difference in this percentage was obtained on comparing the extractions carried out in the cold with those carried out at the boiling point of the solvent*

The preparation of the organs for analysis, the extractions and the estimation of the phospholipins were carried out according to the methods described in a former paper.⁷ The extractions in the cold were conducted as follows: the material was placed in a thick-walled liter bottle, about 500 cc of solvent added, and the bottle shaken vigorously for four periods, of fifteen minutes each, during every twenty-four hours. The extract was then decanted and a fresh supply of the solvent added. This was continued until a fresh supply of solvent yielded no residue when carried to dryness.

I thought it would be of interest also to carry out extractions, first with ether, then with alcohol followed by ether, and compare this extract with that obtained by first extracting with alcohol, then with ether followed by alcohol.

Bact., xii, p 371, 1903, Kennaway and Leathes, *Proc Roy Soc Med*, February, 1909, Leathes and Wedell. *Journ of Physiol*, xxxviii, 1909, Proceedings, p xxxviii.

⁶ *Journ of Physiol*, xiv, p 181, 1896.

⁷ Hanes and Rosenbloom. *Journ of Exp Med*, xiii, p 355, 1911.

The following tables contain the results obtained in this study

TABLE 1

A Extraction with ether followed by alcohol and then ether, at room temperature

NO	WEIGHT OF DRIED HEART MUSCLE	ETHER EXTRACT	ALCOHOL AND ETHER EXTRACT	TOTAL EXTRACT	PERCENTAGE OF LIPINS IN TISSUE	PERCENTAGE OF PHOSPHOLIPINS IN THE LIPIN EXTRACT
	grams	grams	grams	grams		
1	56 4450	7 4140	2 4505	9 8645	17 48	40 54
2	47 1715	6 1230	1 8975	8 0205	17 00	40 80

B Extraction with ether followed by alcohol and then ether in Soxhlet apparatus, at boiling point of solvent

1	61 5260	9 0020	3 9050	12 9070	20 45	41 56
2	61 3020	8 9005	3 6380	12 5385	20 98	41 84

On comparing the above data it is evident that, by extracting the tissue at the boiling point of the solvent, about 3 per cent more lipins are obtained than when the extraction is carried out at the room temperature. This may be due to the breaking up of certain protein-lipin compounds at this temperature. However, the percentage of phospholipins is practically the same in the extract obtained in the cold as compared with that carried out at the boiling point of the solvent.

TABLE 2

A Extraction with alcohol followed by ether and then alcohol, at room temperature

NO	WEIGHT OF DRIED HEART MUSCLE	ALCOHOL EXTRACT	ETHER AND ALCOHOL EXTRACT	TOTAL EXTRACT	PERCENTAGE OF LIPINS IN TISSUE	PERCENTAGE OF PHOSPHOLIPIN IN THE LIPIN EXTRACT
	grams	grams	grams	grams		
1	32 2340	4 4970	1 6805	6 1775	19 16	43 57
2	45 1300	6 7365	2 0810	8 8175	19 54	42 98

B Extraction with alcohol followed by ether and then alcohol in Soxhlet apparatus, at boiling point of solvent

1	44 5830	6 0630	3 0365	9 0995	20 41	42 86
2	44 2490	6 1035	3 3055	9 4909	21 26	42 18

On comparing these extractions, we find that the amount of extract obtained at the boiling point of the solvent is about 10 per cent higher than that obtained in the cold, while the percentage of phospholipins contained in the extract is about the same in both cases. It may also be noted that extraction with ether followed by alcohol and then ether at room temperature gives about 2 per cent less extract when compared with the extract obtained by extraction with alcohol followed by ether and then alcohol at room temperature.

NOTE ON FOLIN'S MICROCHEMICAL METHOD FOR THE DETERMINATION OF UREA

By JOSEPH C BOCK

(From the Nutrition Laboratory of the Carnegie Institution of Washington,
Boston, Massachusetts)

(Received for publication, February 25, 1913)

The total nitrogen in urines as determined by the method of Folin and Farmer¹ can be obtained by determining the ammonia colorimetrically or by titration with $\frac{N}{10}$ or $\frac{N}{100}$ alkali and acid. The urea nitrogen is usually determined colorimetrically,² but the somewhat shorter method of titration was tried in our laboratory. When the urea nitrogen is determined colorimetrically the results are very accurate, but when the titration is used the results obtained are too low. I therefore conducted some experiments to find the cause of this deficiency.

I started by making blank tests with different brands of sodium acetate. Seven grams of sodium acetate were put into a Jena test tube (200 mm. by 20 mm.), a porcelain shot (to prevent bumping), the heat indicator and 1 cc. of 50 per cent acetic acid were added and, in place of the urea solution, 1 cc. of ammonia-free water was used. The stopper with the tube, which acts as reflux condenser, was put in and the tube heated according to the method for ten minutes. After cooling for a short time, the condensing tube was rinsed with 5 cc. of water and, after adding 2 cc. of concentrated sodium hydroxide, air was blown for ten minutes through the apparatus and into an Erlenmeyer flask containing 10 cc. of $\frac{N}{100}$ hydrochloric acid and 40 cc. of water. The air current was of the strength used in all our ammonia and nitrogen determinations.

In each case I found that the amount of acid in the Erlenmeyer flask had increased, which would of course lower the amount of nitrogen. These tests were all made by cooling the tube to room

¹ Folin and Farmer this *Journal*, xi, p 493, 1912

² Folin this *Journal*, xi, p 507, 1912

temperature before blowing the air through. Another series of tests was made without cooling, the mixture in the tube having a temperature of about 45° . The table gives the excess of acid expressed in cubic centimeters of $\frac{N}{100}$ sodium hydroxide

		SODIUM ACETATE BRAND		
		B A	J T B	Kb
At room temperature	{	0 75	0 60	0 6
		0 95	0 60	0 8
		0 65	0 70	0 6
		0 90	0 65	0 7
		1 05		
At 45°C	{		1 6	1 8
			1 7	1 4
			1 5	1 7

These results show that the increase in acidity is greater when the air is blown through the mixture when still hot, that is to say, at a temperature which the liquid will have when the adding of the water, exchange of stoppers, neutralizing, etc., is done in the shortest time possible.

After the mixture is neutralized and cooled below room temperature, a strong smell of acetic acid in the tube is still noticeable. There is a measurable amount of acetic acid vapors standing in the tube which does not condense on cooling and is not neutralized by the sodium hydroxide. The following experiment will prove this statement.

After cooling and adding the water, the sodium hydroxide solution was added and, before blowing the air through the liquid, the air column containing the acetic acid vapors was removed by blowing a gentle current of air in the tube without disturbing the liquid. This could of course not be done in the case of an actual determination. The rest of the experiment was carried out as usual and the acidity was found to be less

Increase in acidity after removing air column on top of liquid	{	0 30
		0 40
		0 20
		0 40
		0 35

RESEARCHES ON PURINES

ON 2-THIO-6,8-DIOXYPURINE AND 2,8-DITHIO-6-OXYPURINE ON THE DESULPHURIZATION OF THIOPURINES ON A NEW METHOD OF PREPARING XANTHINE

NINTH PAPER¹

By CARI O. JOHNS AND ALBERT G. HOGAN

(From the Sheffield Laboratory of Yale University)

(Received for publication, February 25, 1913)

Only one of the three dioxy-monothio-purines theoretically possible has been described, namely, 2,6-dioxy-8-thiopurine². We find that 2-thio-6,8-dioxypurine (V) can easily be prepared in quantity by heating a mixture of 2-thio-4,5-diamino-6-oxypyrimidine (II)³ and urea. The reaction is very smooth, the yield being almost quantitative.

None of the three monoxy-dithio-purines required by theory has hitherto been described. We have also prepared one of these, namely 2,8-dithio-6-oxypurine (III). This purine in good yields by heating a mixture of 2-thio-4,5-diamino-6-oxypyrimidine and thiourea.

W. L. Miller and Liddle⁴ found that when 2-thiouracil was heated in solution of monochloroacetic acid the thio group was converted to uracil-2-thioglycolic acid (XI) which was subsequently hydrolyzed to uracil (XII). G. S. Ge⁵ have shown that thiopyrimidines can be converted to purines by boiling them with an aqueous solution of thiourea. The purines described in this paper form

Ann. Chem. Phys., **xxx**, p. 445, 1898

Liebigs Ann. Chem., **cccxxxi**, p. 75, 1904

Chem. Journ., **xl**, p. 552, 1908

Journ. Amer. Chem. Soc., **xxxv**, p. 1041,

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Some years ago Wheeler and Liddle⁴ found that when 2-thiouracil (X) was boiled in a solution of monochloroacetic acid the thio-pyrimidine was converted to uracil-2-thioglycolic acid (XI) which being unstable was consequently hydrolyzed to uracil (XII) Johnson, Pfau and Hodge⁵ have shown that thiohydantoins can readily be desulphurized by boiling them with an aqueous solution of chloroacetic acid The thiopurines described in this paper form

¹ This *Journal*, xiv, p 1, 1913

² E Fischer *Ber d deutsch chem Gesellsch*, xxxi, p 445, 1898

³ W Traube *Ann d Chem* (Liebig), cccxxx, p 75, 1904

⁴ Wheeler and Liddle *Amer Chem Journ*, xl, p 552, 1908

⁵ Johnson, Pfau and Hodge *Journ Amer Chem Soc*, xxxiv, p 1041, 1912

temperature before blowing the air through. Another series of tests was made without cooling, the mixture in the tube having a temperature of about 45° . The table gives the excess of acid expressed in cubic centimeters of $\frac{N}{100}$ sodium hydroxide.

	SODIUM ACETATE BRAND		
	B A	J T B	Kb
At room temperature	0 75	0 60	0 6
	0 95	0 60	0 8
	0 65	0 70	0 6
	0 90	0 65	0 7
	1 05		
At 45°C		1 6	1 8
		1 7	1 4
		1 5	1 7

These results show that the increase in acidity is greater when the air is blown through the mixture when still hot, that is to say, at a temperature which the liquid will have when the adding of the water, exchange of stoppers, neutralizing, etc., is done in the shortest time possible.

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Increase in acidity after removing air column on top of liquid	{ 0 30
	{ 0 40
	{ 0 20
	{ 0 40
	{ 0 35

thioglycollic acid derivatives which are stable. Thus, hypoxanthine-2-thioglycollic acid (IV) and 6,8-dioxypurine-2-thioglycollic acid (VIII) could be boiled with water for hours without undergoing notable decomposition. On the other hand, when these compounds were boiled with 20 per cent hydrochloric acid, they were hydrolyzed and xanthine (VII) and uric acid (IX), respectively, were obtained. It was found that 6-oxypurine-2,8-dithioglycollic acid (VI) was not desulphurized by boiling for several hours with 20 per cent hydrochloric acid although a small quantity of a dioxypurine-monothioglycollic acid was obtained. This substance was probably 2,6-dioxypurine-8-thioglycollic acid, since, if 6,8-dioxypurine-2-thioglycollic acid had been formed it would have been hydrolyzed to uric acid by the action of the hydrochloric acid. The fact that 6-oxypurine-2,8-dithioglycollic acid is more stable than the monothioglycollic acid derivatives accords with an observation made by Wheeler and Liddle⁶ who found that

uracil-2,6-dithioglycollic acid was not converted to uracil when warmed with hydrochloric acid.

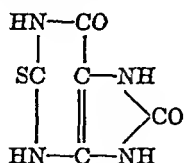
Hitherto the best method of making xanthine has been by the action of nitrous acid on guanine. The xanthine prepared in this manner was highly colored and the color was very difficult to remove. Fischer⁷ used a mixture of fuming hydriodic acid and phosphonium iodide to remove the color from xanthine which had been prepared from guanine. The method described in the following pages gives almost colorless xanthine even without the use of an adsorbent.

As 2-thiohypoxanthine (I) can be made in quantity and is easily converted to xanthine, as herein described, this procedure may be recommended as a method of preparing xanthine in quantity. The stability of the purine-thioglycollic acids renders it probable that these derivatives can be used for various synthetic purposes. These researches will be continued.

⁶ Wheeler and Liddle *Amer Chem Journ*, xl, p 557, 1908

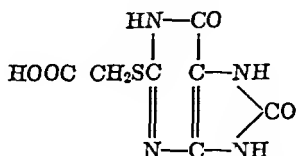
⁷ E. Fischer *Untersuchungen in der Puringruppe*, p 267

EXPERIMENTAL PART

2-Thio-6,8-dioxypurine

Five grams of 2-thio-4,5-diamino-6-oxypyrimidine⁸ and 5 grams of urea were pulverized together and the mixture was heated in an oil bath at 170—180°C for an hour. This treatment gave a hard crust which was dissolved in hot dilute sodium hydroxide. After filtering off a trace of insoluble substance, the hot filtrate was acidified with acetic acid. A precipitate formed immediately. This consisted of minute crystals and weighed 5.4 grams or 93 per cent of the calculated weight. The crystals were very stable and did not melt at 310°C. They were soluble in about 500 parts of boiling water and almost insoluble in cold water. They did not dissolve in alcohol or benzene. They were moderately soluble in dilute ammonia and easily soluble in dilute sodium hydroxide. They gave a brilliant murexide reaction. An ammoniacal solution of the purine gave a precipitate on adding a few drops of silver nitrate solution. The substance was dried at 130°C for analysis.

N	Calculated for	Found	
	C ₆ H ₄ O ₂ N ₂ S	I	II
	30.43	30.53	30.73

6,8-Dioxypurine-2-thioglycollic acid

This compound could be obtained by boiling 2-thio-6,8-dioxypurine in an aqueous solution of monochloroacetic acid but owing to the slight solubility of the dry thiopurine it was prepared as follows

⁸W. Traube *Ann d Chem (Liebig)*, cccxxv, p 75, 1904

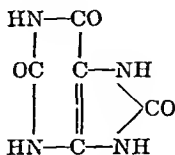
Two grams of 2-thio-6,8-dioxypurine were suspended in 150 cc of hot water and just enough sodium hydroxide to effect solution was added. The purine was then reprecipitated in a finely divided state by adding acetic acid. To this mixture were then added 6 grams of monochloroacetic acid and on boiling for twenty minutes under a return condenser complete solution took place. This solution was evaporated to dryness, the residue was dissolved in dilute ammonia and on acidifying with acetic acid the glycollic acid derivative was obtained as a finely divided precipitate. This dissolved in about 100 parts of boiling water but was difficultly soluble in cold water and did not dissolve in alcohol or benzene. It did not have a melting point but decomposed at about 225°C. It gave a murexide reaction and its ammoniacal solution gave a precipitate on adding silver nitrate. The yield was quantitative. The substance was dried at 130°C for analysis.

N	Calculated for	Found
	$C_7H_4O_4N_4S$	
	23.14	23.40

The ammonium salt of 6,8-dioxypurine-2-thioglycollic acid was obtained when the purine was dissolved in dilute ammonia and the solution was acidified with acetic acid.

N	Calculated for	Found	
	$C_7H_5O_4N_4S$	I	II
	27.02	26.90	26.85

Uric acid

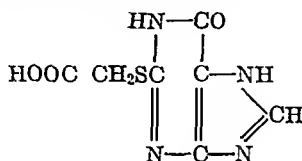


The ammonium salt of 6,8-dioxypurine-2-thioglycollic acid was dissolved in hot 20 per cent hydrochloric acid and the solution was boiled for two hours. Uric acid was obtained. The reaction was quantitative. Uric acid was also obtained from the free purine as follows. A solution of 6,8-dioxypurine-2-thioglycollic acid was prepared as previously described. An equal volume of concentrated hydrochloric acid was then added and the solution was

boiled under a reflux condenser for two hours. The mixture was evaporated to dryness and the residue was dissolved in dilute sodium hydroxide. When the hot solution was acidified with hydrochloric acid, uric acid was obtained in crystalline form. The yield was quantitative.

N	Calculated for	Found	
	$C_7H_4O_2N_4$	I	II
	33.33	33.10	33.20

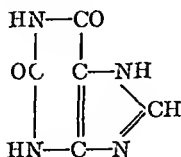
Hypoxanthine-2-thioglycollic acid



Two grams of 2-thiohypoxanthine⁹ were suspended in 150 cc of hot water and sufficient sodium hydroxide to produce solution was added. The thiohypoxanthine was then precipitated in a finely divided state by means of acetic acid. Six grams of monochloroacetic acid were added and the mixture was boiled under a return condenser. In about fifteen minutes the solution was clear and was then evaporated to dryness on a steam bath. The residue was washed with cold water to remove salts and the thioglycollic acid derivative was obtained. This was sparingly soluble in hot and difficultly soluble in cold water and insoluble in alcohol or benzene. It dissolved readily in ammonia or dilute sodium hydroxide. It decomposed at about 240°C forming a violet colored substance. Its ammoniacal solution gave a precipitate with silver nitrate. The substance was dried at 130°C for analysis.

N	Calculated for	Found
	$C_7H_4O_2N_4S$	
	24.77	24.60

2-β-Dioxypurine (Xanthine)



⁹ W. Traube, *Ann. d. Chem.* (Liebig), **cccxxi**, p. 77, 1904.

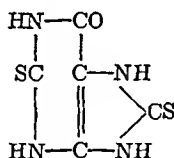
One part of hypoxanthine-2-thioglycollic acid was dissolved in about 75 parts of hot 20 per cent hydrochloric acid and the solution was boiled under a reflux condenser for two hours. After evaporating to dryness the residue was taken up in hot dilute sodium hydroxide and the filtered solution was acidified with acetic acid. The xanthine which precipitated was almost colorless.

In order to prepare xanthine from 2-thiohypoxanthine it is unnecessary to isolate the intermediate thioglycollic acid derivative. When a solution of the latter compound has been prepared, as previously described, an equal volume of concentrated hydrochloric acid is added and the mixture is boiled for two or three hours and then evaporated to dryness. The yield of xanthine is almost quantitative.

N

Calculated for $C_5H_4O_2N_4$	Found
36.84	36.71

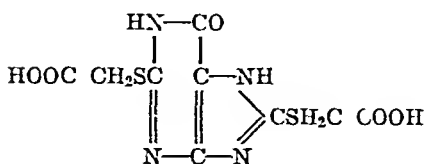
2,8-Dithio-6-oxypurine



Two grams of 2-thio-4,5-diamino-6-oxypyrimidine were pulverized together with 2 grams of thiourea and the mixture was heated in an oil bath at 180°C for an hour. The reaction product was dissolved in hot dilute sodium hydroxide, the solution was filtered to remove a little insoluble material and the hot filtrate was acidified with acetic acid. A granular precipitate was obtained. This was purified by dissolving in dilute ammonia and reprecipitating with acetic acid. The dithiopurine was soluble in about 100 parts of boiling water but did not dissolve in alcohol or benzene. It did not melt at 310°C. It gave a murexide reaction and its ammoniacal solution gave a precipitate on the addition of silver nitrate.

N

Calculated for $C_5H_4ON_4S_2$	I	II
28.60	27.77	27.50

6-Oxypurine-2,8-dithioglycollic acid

One gram of 2,8-dithio-6-oxypurine was dissolved in 75 cc of water containing sufficient sodium hydroxide to produce solution. The purine was then precipitated from the hot solution by adding acetic acid. Six grams of monochloroacetic acid were added and the mixture was boiled until solution took place, whereupon it was evaporated to dryness on the steam bath. The residue was washed in a little cold water. The resulting dithioglycollic acid derivative was easily soluble in hot and moderately soluble in cold water and slightly soluble in hot alcohol. It decomposed with effervescence at about 240°C. It gave a murexide reaction and its ammoniacal solution gave a precipitate with silver nitrate. The substance was dried at 130°C for analysis.

N

Calculated for	Found
$\text{C}_9\text{H}_4\text{O}_4\text{N}_4\text{S}_2$	
17.83	18.50

A portion of the 6-oxypurine-2,8-dithioglycollic acid was boiled with 20 per cent hydrochloric acid for several hours without the formation of uric acid. A difficultly soluble compound was obtained and this gave an analysis for a dioxypurine-monothioglycollic acid. This was probably 2,6-dioxypurine-8-thioglycollic acid since, if 6,8-dioxypurine-2-thioglycollic acid had been formed, it would have been hydrolyzed to uric acid. The substance was dried at 130°C for analysis.

N

Calculated for	Found
$\text{C}_7\text{H}_4\text{O}_4\text{N}_4\text{S}$	
23.14	22.88

RESEARCHES ON PYRIMIDINES PYRIMIDINE-NUCLEOSIDES

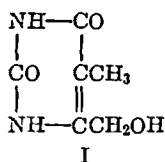
By TREAT B JOHNSON AND LEWIS H CHERNOFF

(SIXTY-FIRST PAPER)

(Contributions from the Sheffield Laboratory of Yale University)

(Received for publication, February 28, 1913)

This paper is a contribution to our knowledge of the constitution of pyrimidine nucleosides. It is our first paper on this subject and includes a description of the synthesis and properties of the simplest nucleoside of thymine (I)



Through the investigations of Levene¹ and his co-workers our knowledge of the constitution of nucleic acids has been advanced to such a degree that we may now regard these interesting substances as composed of characteristic complexes designated by the term *nucleotides*². The latter are compounds consisting of phosphoric acid conjugated with a complex composed of a carbohydrate and a purine or a pyrimidine. In other words, a nucleic acid may be a single nucleotide, as in the case of guanylic³ and

¹ Levene and Mandel *Ber d deutsch chem Gesellsch*, xli, p 1905, Levene and Jacobs *ibid*, xlii, p 2474, xlii, p 2704, xliii, p 3150, xliii, p 1027, Levene and La Forge *ibid*, xliii, p 3164, xlv, p 608, Levene and Jacobs *this Journal*, vii, pp 411, 421, Mandel and Dunham *ibid*, vi, p 85

² Levene and Mandel *loc cit*

³ Levene and Jacobs *Ber d deutsch chem Gesellsch*, xlii, p 2469, *this Journal*, vii, p 421

inosinic⁴ acids (mononucleotides), or be composed of several nucleotides as in the case of yeast nucleic acid. This acid or polynucleotide is a combination of four different nucleotides containing the two purines, guanine and adenine, and the pyrimidines, cytosine and uracil. This nucleotide structure is apparently common to all nucleic acids. Regarding the nature of the union of the individual nucleotides in polynucleotides we have no definite knowledge, but it seems very probable, from the evidence presented, that they are condensed in one molecule in the case of yeast nucleic acid.

The constitution of these nucleotides has also been partly elucidated by the work of these same investigators. It has been shown by Levene⁵ that these compounds may lead, by partial hydrolysis, to the formation of two types of complexes depending upon the experimental conditions employed. For example, it is possible to detach from a nucleotide phosphoric acid alone giving a simpler complex of a sugar and a purine or pyrimidine, viz., *nucleosides*,⁶ or to remove only the nitrogenous nucleus, leaving the phosphoric acid in combination with the carbohydrate. These results are significant and prove that the three constituents of a nucleotide are linked according to the following order—acid sugar, base. Regarding the constitution of the nucleosides of yeast nucleic acid Levene and Jacobs⁷ write as follows: "Die organischen Komplexe der Hefenucleinsäure sind also in zwei Klassen einzuteilen. Die der Purinbasen, welche glykosidartige Verbindungen darstellen, und die der Pyrimidinbasen, deren Konstitution noch nicht ganz aufgeklärt ist."

Direct proof of the presence of these purine-carbohydrate and pyrimidine-carbohydrate combinations has been presented by the isolation of the pentose nucleosides, viz. guanosine, adenosine, uridine and cytidine⁸ from yeast nucleic acid and of guanine-

⁴ Levene and Jacobs *Ber d deutsch chem Gesellsch*, xli, p 2703, xlii, p 335

⁵ Levene and co-workers *loc cit*

⁶ Levene and Jacobs *Ber d deutsch chem Gesellsch*, xlii, p 2475

⁷ *Ibid*, xlii, p 1027

⁸ Levene and co-workers *loc cit*

hexoside⁹ from thymus nucleic acid In chemical terms the nucleotides and their corresponding nucleosides may be expressed by the following empirical formulas

<i>Nucleotides</i>	<i>Nucleosides</i>
O P(OH) C ₅ H ₈ O ₄ C ₅ H ₄ N ₂	C ₅ H ₉ O ₄ C ₅ H ₄ N ₂
II	Adenosine
O P(OH) ₂ C ₅ H ₈ O ₄ C ₅ H ₄ ON ₂	III
Guanylic acid	C ₅ H ₉ O ₄ C ₅ H ₄ ON ₂
IV	Guanosine
O P(OH) C ₅ H ₈ O ₄ C ₅ H ₄ ON ₂	V
Inosinic acid	C ₅ H ₉ O ₄ C ₅ H ₄ ON ₂
VI	Inosine
O P(OH) C ₅ H ₈ O ₄ C ₄ H ₄ ON ₂	VII
Cytidine-nucleotide	C ₅ H ₉ O ₄ C ₄ H ₄ ON ₂
VIII	Cytidine
O P(OH) ₂ C ₅ H ₈ O ₄ C ₄ H ₃ O ₂ N ₂	IX
Uridine-nucleotide	C ₅ H ₉ O ₄ C ₄ H ₃ O N
X	Uridine
	XI

In their fifth paper on yeast nucleic acid entitled "Die Struktur der Pyrimidin-Nucleoside" Levene and La Forge¹⁰ have presented conclusive evidence that the pyrimidine nucleosides, uridine and cytidine, are combinations of uracil and cytosine, respectively, with the pentose sugar ribose In fact, the exact relationship of these two nucleosides was established by this observation, since it had already been shown that the pyrimidines are linked, in these two nucleosides, to the carbohydrate in a similar manner This was established by the fact that cytidine is transformed by the action of nitrous acid into uridine¹¹ Regarding the nature of this nucleoside union and the position substituted by the sugar in the pyrimidine ring, sufficient data have not been presented to enable us to express structurally the exact constitution of these compounds Levene and La Forge¹² conclude, however, from good evidence, which we will not discuss in this paper, that this linking is of a glucosidic nature and that the carbohydrate may be joined

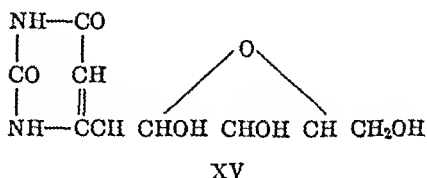
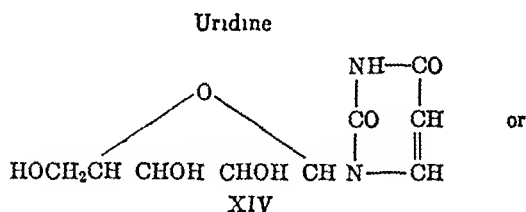
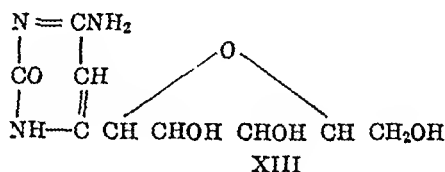
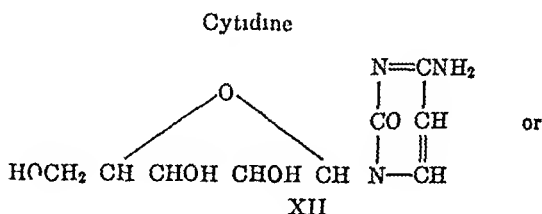
⁹ Levene and Jacobs *this Journal*, xii, p 377

¹⁰ *Ber d deutsch chem Gesellsch*, xlv, p 608

¹¹ Levene and Jacobs *Ber d deutsch chem Gesellsch*, xlv, p 1027

¹² *Loc cit*

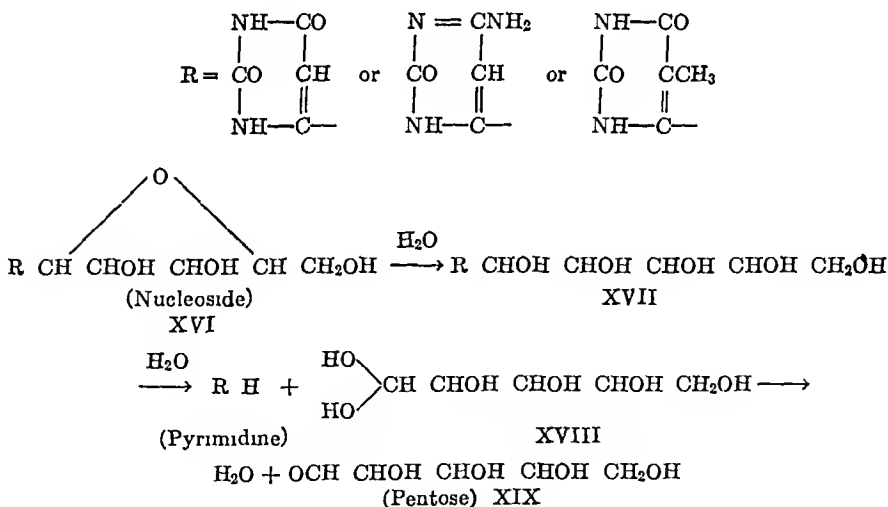
to the pyrimidine in one of two positions, viz, the 3- or 4-position of the ring. If these assumptions be correct then the constitution of cytidine and uridine may be expressed by the following structural formulas



The remarkable stability of these substances, in the presence of hydrolytic agents, seems to indicate that the point of union of the carbohydrate is in position 4 of the ring, as represented by formulas (XIII) and (XV), rather than in position 3 as represented by formulas (XII) and (XIV). Whether the pyrimidine-nucleosides (hexosides) of thymus nucleic acid correspond in constitution to the nucleosides under discussion, has not been established.

The recent work of Levene and Jacobs¹³ on thymus nucleic acid seems to indicate that the determination of the constitution of this acid is a far more complex problem than that presented by yeast nucleic acid

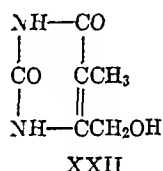
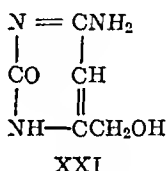
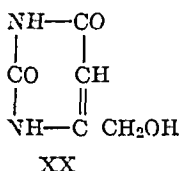
The synthetical work now in progress and also that discussed in this paper, has developed from the assumption that the pyrimidines, in their corresponding nucleosides, are linked to the carbohydrates at position 4 and that this linkage is between two carbon atoms as represented by formulas (XIII) and (XV). A nucleoside may be considered, therefore, as an addition-product of a pyrimidine and a sugar. The formation of ribose from such a complex, by hydrolysis, would then involve, theoretically, two distinct changes, viz a rupture of the furane ring forming the glucoside (XVII) and finally a cleavage of the carbohydrate (XIX) from the pyrimidine. These various changes may be expressed as follows



Judging from what has already been observed in this laboratory, it would be predicted that a union of this character would be extremely stable and very resistant to the action of hydrolytic agents

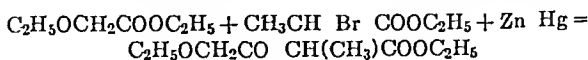
¹³ This *Journal*, 41, p 411

We can conceive, therefore, of an homologous series of these pyrimidine-nucleosides, each differing from its next member in the series by $-\text{CHOH}$. The physical properties of members of such a series would be expected to undergo a gradual change while, on the other hand, the chemical properties would be the same. Consequently if we successively removed a $-\text{CHOH}$ from the chain of the sugar molecule we would finally obtain the prototype of the series or the simplest nucleoside of this type. It would still retain the same glucosidic linking. The three pyrimidines, which would result by this process, are represented by formulas (XX), (XXI) and (XXII).



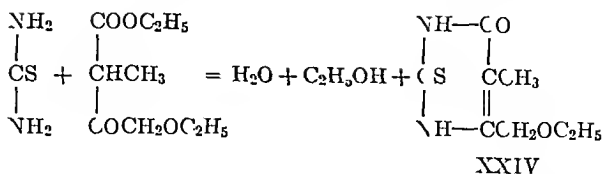
We have absolutely no knowledge of the chemical properties of hydroxypyrimidines of this type and it was not until recently that the writer was able to undertake their investigation. We are now able, however, to contribute data regarding their chemical behavior, which we believe to be of great biochemical interest. We shall now discuss the synthesis of the simplest nucleoside of thymine, viz. 2,6-dioxy-4-hydroxymethyl-5-methylpyrimidine (XXII).

The new ketone ester, ethyl methylethoxyacetoacetate (XXIII) was first prepared by the condensation of ethyl α -bromopropionate with ethyl ethoxyacetate in the presence of zinc-amalgam.

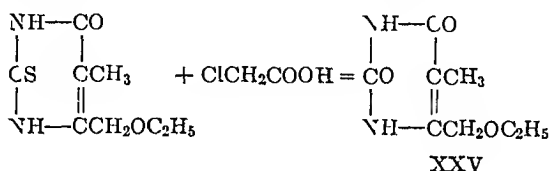


XXIII

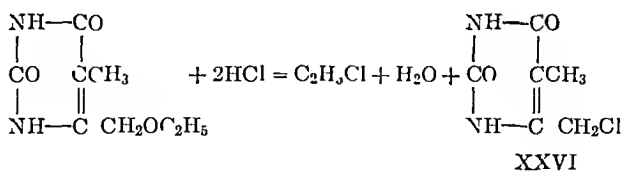
This ester (XXIII) was then digested in alcohol with thiourea and sodium ethylate when a pyrimidine condensation was effected and 2-thio-4-ethoxymethyl-5-methyl-6-oxypyrimidine (XXIV) was formed. This reaction is expressed by the following equation



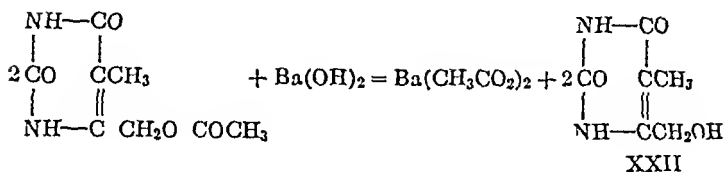
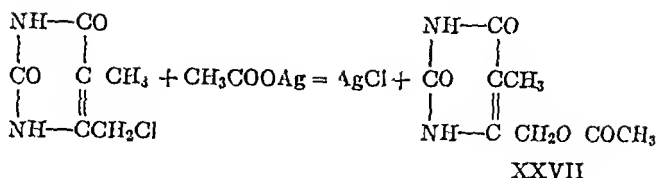
This thiopyrimidine (XXIV) was then digested with chloroacetic acid when it was desulphurized practically quantitatively and the corresponding oxypyrimidine (XXV) was formed



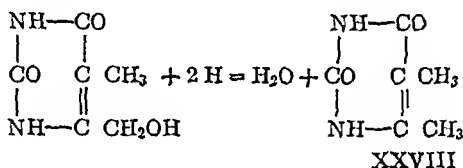
This ethoxypyrimidine (XXV) was then heated with concentrated hydrochloric acid. By means of this reagent the ethyl group was detached in the form of ethylchloride and the pyrimidine was converted smoothly into the chloromethylpyrimidine (XXVI). This interesting change is represented by the following equation



The halogen in this pyrimidine (XXVI) is very reactive and is easily removed by alkaline hydrolysis. For example the pyrimidine was transformed, almost quantitatively, into the corresponding acetate (XXVII) when digested in aqueous solution with the required amount of silver acetate. This pyrimidine possessed characteristic properties and underwent a smooth hydrolysis, when digested with barium hydroxide solution, forming the corresponding alcohol (nucleoside) (XXII). These final changes in this synthesis are represented as follows



The constitution of this nucleoside (XXII) was established by the fact that it was transformed quantitatively into 2,6-dioxy-4-5-dimethylpyrimidine¹⁴ (XXVIII) by reduction with hydriodic



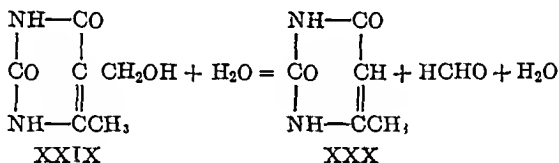
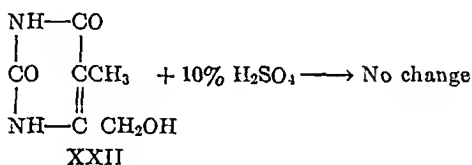
acid and red phosphorus. Starting with the two aliphatic esters, ethyl α -bromopropionate and ethyl ethoxyacetate, our synthesis therefore involves six operations, and in every step, except the first, the yields are excellent. We are now investigating this unsatisfactory step in order to determine the cause of the low yield.

We now find that this simple nucleoside of thymine (XXII) is extremely stable in the presence of acids and does not undergo hydrolysis with formation of thymine and formaldehyde when heated with sulphuric acid. It has been shown in the papers by Levene and his co-workers¹⁵ that the carbohydrate is cleaved from the pyrimidine-nucleosides by heating with 10 per cent sulphuric acid at 125°. Our pyrimidine was recovered unaltered after heating with sulphuric acid, of this same strength, for three hours at 125–130°. This result is all the more interesting since

¹⁴ Schlenker *Ber d deutsch chem Gesellsch*, xxxiv, p 2812, Wheeler and Merriam *Amer Chem Journ*, xlix, p 488

¹⁵ *Loc cit*

Kircher¹⁶ has recently shown that the isomeric pyrimidine, 2,6-dioxy-4-methyl-5-hydroxymethylpyrimidine (XXIX) is transformed quantitatively into 4-methyluracil (XXX) and formaldehyde simply by heating in aqueous solution. From a chemical standpoint these results are extremely interesting and very significant. Whether the linking between the carbohydrate complex and the pyrimidine at position 4 will become more unstable as we increase the length of the sugar chain, must be decided by further investigations. We shall endeavor to develop methods of synthesizing some of these higher homologues. The corresponding hydroxy derivatives of uracil (XX) and cytosine (XXI) will also be investigated.



EXPERIMENTAL PART

*Ethyl methylethoxyacetoacetate*¹⁷



This interesting β -ketone ester was prepared by condensing ethyl α -bromopropionate with ethyl ethoxyacetate by means of zinc-amalgam. The method of procedure was essentially as follows. Molecular proportions of the bromopropionate (84.4 grams)

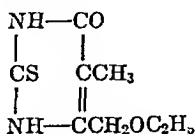
¹⁶ *Ann d Chem* (Liebig), ccclxxv, p 293

¹⁷ This new method of synthesis, which we have employed for the preparation of this ester, has been applied successfully in several other cases. The reaction is being investigated and the results of the investigation will be published in future papers. We are also using zinc-amalgam as a reagent in other lines of investigation and have obtained interesting results, which we soon hope to be able to present for publication. (T B Johnson)

and the acetate (56.0 grams) were placed in a dry flask and 40.8 grams of dry amalgamated zinc suspended in the liquid. The flask was then connected with a return condenser and finally heated on the steam bath. At first there was no evidence of any reaction, but after warming a few minutes a violent reaction began and became so vigorous that it was necessary to plunge the flask into ice water at intervals to avoid too great heat. After the violent reaction was over the flask was then heated on the steam bath for about twelve hours in order to thoroughly complete the reaction. We obtained a dark brown, syrupy fluid. This was then transferred to a separatory funnel and shaken with an excess of water when we obtained a heavy precipitate, which was immediately dissolved by addition of cold dilute hydrochloric acid. We obtained in this manner a transparent red oil, which was separated from the acid solution and finally dissolved in ether. This ether solution was then thoroughly cooled with crushed ice and washed repeatedly with a cold, dilute solution of sodium hydroxide. The β -ketone ester was removed by this treatment and the alkaline solutions finally combined and acidified (cold) with cold, dilute hydrochloric acid. The ketone ester separated at once and was dissolved in ether. After thorough drying over anhydrous calcium chloride the ether was removed and the ester purified by distillation under diminished pressure. It practically all distilled at 116° at 24 mm. The yield of purified material was 7.5 grams. Molecular weight determination by the ebullioscopic method

I	0.6731 gram substance in 15.56 grams of benzene gave $\Delta_0 = 619^\circ$	
	Calculated for $C_9H_{12}O_4$	Found
Molecular Weight	188	186

2-Thio-4-ethoxymethyl-5-methyl-6-oxypyrimidine

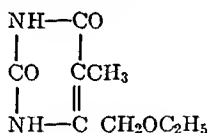


This pyrimidine was formed by condensation of the preceding β -ketone ester with thiourea in the presence of sodium ethylate. The following proportions were used: 7.4 grams of the ketone

ester, 3.0 grams of thiourea and 1.8 grams of metallic sodium. The sodium was dissolved in a small volume of absolute alcohol, the thiourea and ketone ester dissolved in the solution and the mixture then digested on the steam bath for about four hours. The sodium salt of the pyrimidine began to form almost immediately on heating, and deposited as a brown powder. After completion of the reaction the alcohol was then evaporated and the residue dissolved in a small volume of hot water and the solution filtered. On acidifying this solution (cold) with glacial acetic acid the pyrimidine separated at once in a crystalline condition. It was purified by crystallization from boiling 95 per cent alcohol and separated, on cooling, in hexagonal tables, which melted at 191–192° to a clear oil without decomposition. The yield of purified pyrimidine was 2.3 grams. The pyrimidine is very soluble in hot water and hot alcohol and difficultly soluble in cold.

NITROGEN DETERMINATION (Kjeldahl)

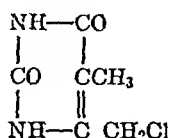
	Calculated for $C_8H_{11}O_2N_2S$	Found
N	13.86	13.90

2,6-Dioxy-4-ethoxymethyl-5-methylpyrimidine

One and three-tenths grams of 2-thio-4-ethoxymethyl-5-methyl-6-oxypyrimidine and two molecular proportions of chloroacetic acid (1.1 grams) were dissolved in 30 cc. of water and the solution boiled for five hours. The solution was then allowed to cool slowly when this pyrimidine separated in beautiful, arborescent crystals. The compound was purified by crystallization from hot water and melted at 220° to a clear oil. It is soluble in hot alcohol. The yield was 1.1 grams.

NITROGEN DETERMINATION (Kjeldahl)

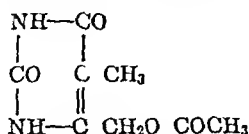
	Calculated for $C_8H_{12}O_2N_2$	Found
N	15.05	15.02

2,6-Dioxy-4-chlormethyl-5-methylpyrimidine

This chlorpyrimidine was prepared by heating the preceding pyrimidine with hydrochloric acid. One gram of the ethoxy-pyrimidine and 20 cc of concentrated hydrochloric acid were heated in a bomb tube for three hours at 125–130°. When the tube was opened ethyl chloride was identified and a yellow solution was obtained. This was then concentrated on the steam bath and cooled when this chlorpyrimidine separated in the form of plates, which melted at 243° to a clear red oil. On evaporating the filtrate to dryness more of the same compound was obtained. The pyrimidine was purified by crystallization from boiling water and separated, on cooling, in stout prismatic crystals or blocks which melted at 244–245° to a clear oil without decomposition. It gave a strong test for chlorine. The dust from this pyrimidine irritates the nose causing violent sneezing and finally a severe headache. The yield was excellent.

NITROGEN DETERMINATION (Kjeldahl)

N	Calculated for	Found
	$\text{C}_6\text{H}_7\text{O}_2\text{N}_2\text{Cl}$	
	16.04	15.81

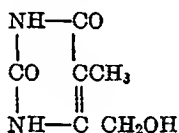
The acetate of 2-6-dioxy-4-hydroxymethyl-5-methylpyrimidine

Two and four-tenths grams of pure silver acetate were dissolved in about 200 cc of hot water and 2.086 grams of the above chlorpyrimidine added to the hot solution. There was an immediate reaction, the pyrimidine dissolved and silver chloride deposited. This solution was finally boiled for two hours in order to complete the reaction. After filtering from silver chloride the solution was then evaporated to complete dryness and the residue dissolved

again in hot water and the slight amount of silver salt in solution decomposed by treatment with hydrogen sulphide. After digesting with bone coal the solution was then filtered and concentrated to a small volume. On cooling, the acetylpyrimidine separated in minute crystals. It was difficultly soluble in hot water and cold 95 per cent alcohol. It was purified by crystallization from 95 per cent alcohol and deposited in microscopic, corpuscular crystals, which melted at 260–261° with effervescence. The yield of purified material was about 1.5 grams.

NITROGEN DETERMINATION (Kjeldahl)

	Calculated for $C_8H_{10}O_4N_2$	Found
N	14.14	14.34

2,6-Dioxy-4-hydroxymethyl-5-methylpyrimidine

This interesting pyrimidine was obtained by saponification of the above acetate. Five grams of crystallized barium hydroxide and one gram of the acetylpyrimidine were dissolved in the least possible volume of hot water and the solution boiled for one hour. The solution was then saturated with carbon dioxide gas in order to precipitate the barium as carbonate, and the solution finally filtered. The solution was then evaporated to dryness when the crude hydroxypyrimidine was obtained as an amber colored crystalline residue. This was purified by crystallization from hot water and separated, on cooling, in distorted needles, which melted at 224–225° with decomposition.

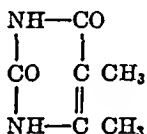
NITROGEN DETERMINATION (Kjeldahl)

	Calculated for $C_8H_{10}O_4N_2$	Found
N	17.94	17.82

An attempt to convert this pyrimidine into thymine by hydrolysis with 10 per cent sulphuric acid was unsuccessful. One half of a gram of the pyrimidine was suspended in 25 cc. of 10 per cent sulphuric acid and then heated in a bomb tube for three hours at

120–130° When the tube was opened there was no pressure and no evidence that the pyrimidine had undergone any change. The sulphuric acid was exactly precipitated as barium sulphate by addition of the required amount of barium hydroxide, and the barium-free filtrate then evaporated to dryness. We obtained a crystalline substance, which was purified by crystallization from hot water. It separated in the form of distorted prisms which melted at 223–224° with effervescence. It did not resemble thymine in any of its properties and was identified as the unaltered hydroxypyrimidine. We recovered all of the pyrimidine and used part of it for the following experiment.

The conversion of 2,6-dioxy-4-hydroxymethyl-5-methylpyrimidine into 2,6-dioxy-4,5-dimethylpyrimidine



One-tenth of a gram of the hydroxypyrimidine was dissolved in 5 cc of hydriodic acid (sp gr 1.7) and the solution, after the addition of a few milligrams of red phosphorus, was boiled for four hours. After cooling, the solution was then diluted with water, filtered and then an excess of dry silver carbonate stirred into the solution in order to remove all the iodine, hydriodic and phosphoric acids. After filtering, the excess of silver was then precipitated as sulphide and the solution then concentrated on the steam bath and finally allowed to cool. Dimethyluracil separated in the form of prismatic crystals, which melted at 296° when heated rapidly. If heated slowly the substance melted at 292–294° with partial decomposition. A mixture of this compound with 4,5-dimethyluracil¹⁸ melted at exactly the same temperature.

¹⁸ Schlenker *loc cit*, Wheeler and Merriam *loc cit*

STUDIES ON THE INTERMEDIARY METABOLISM OF AMINO-ACIDS

By H D DAKIN

(*From the Herter Laboratory, New York*)

(Received for publication, March 1, 1913)

The object of the following paper is to record experiments concerning the intermediary metabolism of the amino-acids derived from proteins. By utilizing the method of liver perfusion, Embden and others have shown that certain amino-acids, particularly tyrosine and phenylalanine, may give rise to acetoacetic acid. Confirming these results, Baer and Blum have been able to demonstrate an increased excretion of acetoacetic acid and β -hydroxybutyric acid when phenylalanine and tyrosine are given to human diabetics. On the other hand, by employing glycosuric animals, rendered diabetic by pancreas extirpation or by phlorhizin administration, other amino-acids have been shown to be capable of furnishing glucose (Knopf, Glassner and Pick, Embden, Lusk, Ringer and others) ¹

The value of these methods of investigation can hardly be questioned, although the interpretation of the results is often difficult. The formation of either acetoacetic acid or glucose from amino-acid can only take place as the result of intricate molecular rearrangements and much work is necessary before an adequate analysis of the reactions can be undertaken. It is of course open to question as to how closely the normal path of catabolism resembles that observed under abnormal conditions, but it may be safely asserted that an accurate knowledge of what may happen to a food substance under any conditions, no matter how far removed from normal, is likely to be helpful in gradually filling in the intricate mosaic of metabolic reactions.

¹ For the literature of the subject, the reader is referred to the article on Phlorhizinglukosurie by Lusk *Ergeb d Physiol*, vii, p 315, 1912

At the present time, by use of the methods referred to, we know that phenylalanine and tyrosine yield acetoacetic acid freely, leucine less readily, while its formation from isoleucine and histidine is somewhat doubtful. On the other hand, glycine, alanine, aspartic and glutamic acids are known to be capable of leading to sugar synthesis in the glycosuric organism. It would seem, therefore, as if a relatively sharp separation might be made between the amino-acids capable of either acetoacetic acid or glucose formation.

It seemed very desirable that these investigations should be extended. Accordingly, the behavior in the glycosuric animal of all the other amino-acids known to occur in proteins has been investigated and also the effect of a number of them on perfusion through a surviving liver.

It has been found that serine, cysteine, proline², ornithine and arginine are all capable of yielding large amounts of sugar when given to glycosuric dogs. Valine³, leucine, isoleucine, lysine, histidine, phenylalanine and tryptophane yield relatively little or no sugar. Ornithine, lysine, arginine, proline, tryptophane and di-iodotyrosine do not yield acetoacetic acid in marked amounts when added to blood perfusing a dog's liver.

As will be seen from the experimental details, it is difficult to definitely state that administration of an amino-acid yields absolutely no glucose, but it is relatively easy to distinguish between those that yield large amounts and those which yield little or none.

It seems not unlikely that the administration to a glycosuric animal of relatively large amounts of an amino-acid which in itself is not convertible into glucose may however lead to an apparent small glucose excretion through its mass action in displacing other amino-acids. The writer is therefore unwilling to attach much significance to results leading to an apparent increased glucose excretion of less than 20 per cent of the amino-acid given.

By combining the new results with those previously obtained, it would appear that certain generalizations are possible. The following table represents the collected data.

² The results in the case of proline have been recently published *this Journal*, xii, p 513, 1913

³ α -Hydroxyisovaleric acid under similar conditions yields little or no glucose

SUBSTANCE	INCREASED OLUCOSE EXCRETION WHEN GIVEN TO GLYCOSURIC DOG*	INCREASED ACETGACETIC ACID WHEN PERFUSED THROUGH SURVIVING LIVER
Glycine	+	-
Alanine	+	-
Serine	+	-
Cysteine	+	-
Aspartic Acid	+	-
Glutamic Acid	+	-
Ornithine	+	-
Proline	+	-
Valine	-	-
Leucine	-	+
Isoleucine	-	-
Lysine	-	-
Arginine	+	-
Histidine†	-	+†
Phenylalanine	-	+
Tyrosine	-	+
Di-iodotyrosine	-	-
Tryptophane	-	-

* Only those amino-acids which yield relatively large amounts of glucose are recorded as positive. Doubtful cases are recorded as negative.

† See p. 328

‡ The increased acetoacetic acid is probably not directly derived from histidine (p. 328)

The following conclusions may be tentatively drawn

1 The amino-acids derived from proteins which may yield glucose freely in the glycosuric organism are all those containing two, three, four and five carbon atoms, except valine

2 Arginine is the only amino-acid with more than five carbon atoms which may furnish glucose freely, and in this case the sugar evidently comes from the ornithine moiety with five carbon atoms, into which it may be converted by the action of arginase

3 All the straight-chain amino-acids yield sugar with the exception of lysine

4 The amino-acids with branched chains including valine, leucine and isoleucine furnish little or possibly no sugar

5 Proline is the only cyclic amino-acid yielding much glucose. Undoubtedly opening of the ring is the first step in its breakdown. None of the aromatic amino-acids yields glucose in considerable amount

6 The close structural relations between ornithine, (arginine), proline and glutamic acids, all of which yield approximately equiv-

alent amounts of glucose, would indicate that their catabolic paths may be similar

7 Certain amino-acids, including tryptophane, lysine and valine, yield neither acetoacetic acid nor glucose in considerable amount

8 The fact that, while alanine and serine yield large amounts of glucose in the glycosuric animal, phenylalanine, tyrosine and tryptophane, all of which contain an alanine side-chain, do not, is clearly in harmony with the view that the side-chain containing three carbon atoms of these amino-acids is broken up. This may be regarded as evidence in support of the writer's view as to the mechanism of acetoacetic acid formation from phenylalanine and tyrosine, in which it was suggested that two of the four carbon atoms of acetoacetic acid were derived from the side-chain and two from the nucleus

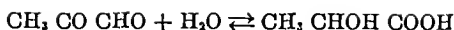
In conclusion, a few words may be said upon the possible mechanism of glucose formation from amino-acids, duly recognizing the fragmentary character of the evidence. The fact that, according to the careful experiments of Lusk and Ringer,⁴ glycine and alanine are quantitatively converted into glucose in the glycosuric organism would seem to inevitably point to the occurrence of a mechanism in the body for the reduction of the carboxyl group. Without such a change the fairly direct conversion of glycine and alanine into glucose is of course inconceivable, but at first sight the possibility of such a reaction is hard to picture

The removal of the carboxyl group by a process of α -oxidation with formation of a lower aldehyde is incompatible with the quantitative glucose formation

In searching for some more likely scheme, reference may be made to a new type of hydrolysis by an enzyme recently observed by Dudley and the writer. It was found that α -ketonic aldehydes, such as methyl glyoxal or phenyl glyoxal were converted into the corresponding α -hydroxyacids, namely, lactic and mandelic acids, with remarkable speed. If some such type of reaction should prove to be reversible, a solution might be found to the difficult problem of the mechanism of the reduction of the carboxyl group of alanine and similar acids.⁵

⁴ *Zeitschr f physiol Chem*, lxxvi, p 106, 1910

⁵ Since the above was written Dudley and the writer have succeeded in showing the reversibility of this reaction *in vitro*



The ready formation of methyl glyoxal from sugar *in vitro*, together with the fact that lactic acid as shown by Lusk is so readily converted into glucose in the diabetic organism, would lend support to the hypothesis of the intermediate formation of lactic acid and methyl glyoxal in the synthesis of glucose in the glycosuric animal. Moreover, the fact that both *d*- and *l*-alanine and probably *d*- and *l*-lactic acid are quantitatively converted into dextro-rotatory glucose would lead to the inference that the asymmetry of the α -carbon atoms in alanine and lactic acid is lost in the process of glucose synthesis. The intermediate formation of the methyl glyoxal would furnish an adequate explanation of this change since, on reconversion into sugar or lactic acid, asymmetry of the carbon atoms would be regained by a process of asymmetric synthesis.

Ringer and Lusk have shown that the amount of glucose obtainable from aspartic and glutamic acids in the glycosuric animal corresponds to about that derivable from three of the carbon atoms in each acid. They picture the hypothetical conversion of aspartic acid into hydracrylic acid and of glutamic acid into glyceric acid. To the writer it appears more probable that aspartic acid may yield either alanine or lactic acid. Some analogy for the removal of carbon dioxide from aspartic acid is found in its reduction to propionic acid by heating with hydriodic acid. The conversion of cysteine into taurine may also be cited as an example of the removal of the carboxyl group from an amino-acid.

The formation of a serine or alanine nucleus from glutamic acid would seem intelligible on the basis of β -oxidation as occurring in fatty acids, for glutamic acid is relatively a strong acid. What is true of glutamic acid may hold for proline and ornithine.

Finally, the fact that the amino-acids with branched chains do not readily form glucose in the glycosuric organism may be referred to the difficulty of lactic acid formation with its straight chain of three carbon atoms.

	PERIOD	EXPERIMENT I	EXPERIMENT II
Arginine	II	0 068	0 052
	III	0 278	0 187
	IV	0 111	0 071
Lysine	II	0 052	0 190
	III	0 226	0 782
	IV	0 106	0 171

It is clear that in every case a small but definite rise in the basic nitrogen of the urine followed the injection of arginine and lysine. In the second lysine experiment, it amounted to about one-fifth of the nitrogen contained in the lysine given. In the other cases it was materially less.

Histidine The crystalline hydrochloride was prepared by Frankel's method in considerable quantity. In the earlier experiments, the hydrochloride was decomposed by an equivalent of sodium bicarbonate and injected intravenously. A minimal amount of ether was given to the dog as anaesthetic during the exposure of the vein. Under these conditions a marked increase in glucose output followed the administration of histidine, but later experience would indicate that at least some of this glucose did not originate from the base but was due to the anaesthetic and to metabolic disturbance following the intravenous injection. Therefore these results have been discarded. Later experiments, in which neutral histidine acetate, free from sodium chloride, was administered subcutaneously, showed in every case a slight increase in glucose output except in the case of one animal that had extraordinarily low sugar output throughout the experiment. A final decision, as to whether histidine really yields glucose in the diabetic animal, does not seem possible at present. Examination of the urines showed that from 2 to 7 per cent of the histidine was excreted unchanged. It will be noted that no regular increase in acetoacetic acid excretion was observed so that it appears unlikely that the slightly increased acetoacetic acid formation found on perfusing a surviving liver with blood containing histidine comes directly from the histidine. Histidine and lysine salts appear to be injurious to the perfused liver as judged by its rather congested and dark-colored appearance.

Phenylalanine The synthetic acid was employed No homogentisic acid was detected in the urine An increased excretion of acetoacetic acid and β -hydroxybutyric acid was evident

Tyrosine The active amino-acid from casein was used No increase in glucose excretion but a distinct increase in acetoacetic acid was observed, thus confirming the results of Lusk and Ringer

Tryptophane was prepared by Hopkins and Cole's method A slight increase in glucose excretion was observed but no acetoacetic acid formation About three grams of kynurenic acid were separated from the urine by simple acidification with sulphuric acid An attempt to estimate unchanged tryptophane in the urine by means of precipitation with mercuric sulphate after removal of kynurenic acid indicated the presence of about 3 grams of the amino-acid

The metabolism of tryptophane in the glycosuric animal appears to resemble closely its behavior in the normal animal

TABLE I

SUBSTANCE	NITROGEN OF SUBSTANCE	WEIGHT OF DOG	PERIOD	TOTAL NITROGEN	GLUCOSE	C/N	EXTRA GLUCOSE	ACETOACETIC ACID	β HYDROXY- BUTYRIC ACID
Serine, 1.29 gms	1.59	6	I			2.90			
			II	5.92	15.44	2.87		0.259	
			III	6.67	24.68	3.70		0.227	
			IV	5.60	17.02	3.03	11.0		
Cysteine, 14.88 gms	1.72	16	I			3.73			
			II	7.83	28.32	3.62		0.217	
			III	10.23	38.12	3.72		0.154	
			IV	8.73	29.73	3.40	8.4	0.136	
			V	8.04	26.54	3.30		0.076	
Cysteine, 15.73 gms	1.82	11	I			3.41			
			II	6.11	20.32	3.32		0.053	
			III	7.43	30.36	4.09		0.054	
			IV	7.35	24.90	3.39	12.2	0.080	
Valine, 20 gms	2.39		I			3.46			
			II	4.04	13.18	3.26		0.123	
			III	5.20	12.14	2.33		0.043	
			IV	4.38	13.86	3.16	1.8	0.253	

TABLE I—Continued

SUBSTANCE	NITROGEN OF SUBSTANCE	WEIGHT OF DOO	PERIOD	TOTAL NITROGEN	GLUCOSE	C/N	EXTRA GLUCOSE	ACETOACETIC ACID	β HYDROXY- BUTYRIC ACID
Valine, 7 gms	0 84	10	I			3 27	0 5		
			II	4 04	13 60	3 36		0 730	
			III	4 62	13 85	3 00		0 440	
			IV	3 69	11 52	3 12		0 512	
Valine, 9 27 gms	1 10	10	I			3 61	0		
			II	5 32	17 48	3 29		1 921	
			III	5 48	12 56	2 29		1 368	
			IV	4 98	16 80	3 37		0 781	
			V			3 40			
Isoleucine, 15 gms	1 60	12	I	3 27	13 02	3 99	3 8	0 081	
			II	4 35	14 36	3 30		0 126	
Isoleucine, 15 gms	1 60		III	3 50	12 01	3 43		0 281	
			IV	3 57	12 25	3 43	2 9	0 347	
			V	4 99	14 48	2 90		0 306	
Leucine, 15 grams	1 60	13	I			3 50	4 0		
			II	5 31	18 38	3 46		0 471	0 735
			III	6 69	21 72	3 25		0 459	0 520
Ornithine, 9 68 grams	2 06	13	I			3 21	5 5		
			II	3 37	10 78	3 20		0 168	
			III	4 95	15 08	3 04		0 549	
			IV	3 69	11 52	3 12		0 512	
Lysine, 12 69 grams	2 43	10	I			3 52	0		
			II	3 19	10 71	3 36		0 042	
			III	6 12	12 99	2 12		0 045	
			IV	4 83	13 92	2 88		0 063	
Lysine, 16 43 grams	3 15	17	I			3 35	3 3		
			II	13 04	43 41	3 33		0 140	
			III	13 54	38 85	2 87		0 150	
			IV	13 34	43 52	3 26		0 198	
Arginine, 15 grams	4 83	9	I			2 84	10 4		
			II	3 47	10 12	2 91		0 068	
			III	6 53	18 54	2 84		0 278	
			IV	5 37	12 13	2 26		0 110	

TABLE I—Continued

SUBSTANCE	NITROGEN OF SUBSTANCE	WEIGHT OF DOG	PERIOD	TOTAL NITROGEN	GLUCOSE	C/N	EXTRA GLUCOSE	ACETOACETIC ACID	β HYDROXY- DUTIRIC ACID
Arginine, 6.89 grams	2.22	kgm 7	I	2.99	8.17	2.73	2.4	0.052	
			II	4.83	12.05	2.49		0.187	
			III	3.91	8.05	2.06		0.071	
Arginine, 12.58 grams	4.05	9	I			3.08	8.5		
			II	5.22	14.41	2.76			
			III	8.27	20.80	2.51			
Histidine, 13.2 grams	3.58		I			3.53	4.4		
			II	3.41	11.97	3.51		0.037	
			III	6.36	14.58	2.29		0.135	
			IV	3.57	11.01	3.24		0.044	
			V	3.20	10.14	3.15		0.078	
Histidine, 9.9 grams	3.37	6	I			3.81	4.6		
			II	3.87	14.01	3.62		0.056	
			III	3.92	13.55	3.45		0.008	
			IV	4.85	12.43	2.56		0.009	
			V	4.24	13.50	3.18		0.007	
Histidine, 11.60 grams	3.95	6	I	3.21	10.27	3.20	3.2	0.144	
			II	5.18	11.52	2.22		0.214	
			III	5.14	12.05	2.34		0.141	
Histidine, 11.1 grams	3.01	12	I			3.38	2.6		
			II	6.74	21.02	3.13		0.026	0.10
			III	10.00	25.33	2.53		0.206	0.27
			IV	7.26	21.88	3.02		0.489	1.08

TABLE II

SUBSTANCE	NITROGEN OF SUBSTANCE	WEIGHT OF DOO	PERIOD	TOTAL NITROGEN	GLUCOSE	C/N	EXTRA GLUCOSE	ACETOACETIC ACID	β HYDROXY- BUTYRIC ACID
		kgm							
Histidine, 8.38 grams	2.46	7	I			3.99			
			II	1.45	5.68	3.91		0.016	
			III	2.65	5.76	2.17	0	0.009	
			IV	2.51	4.52	1.80		0.014	
Histidine, 11.18 grains	3.28	7	I			3.46			
			II	4.24	14.80	3.49		0.260	
			III	6.47	16.72	2.59	2.2	0.127	
			IV	5.07	14.14	2.79		0.315	
Phenylalanine, 11 grams	0.93	7	I	3.48	12.67	3.64		0.734	0.97
			II	4.55	15.10	3.32	1.2	2.397	2.52
			III	3.80	12.56	3.30		1.821	1.46
			IV	3.61	12.66	3.51			1.56
Tyrosine, 20 grams	1.55	18	I			3.05			
			II	8.69	30.25	3.48		0.140	0.31
			III	9.32	32.16	3.45	0	0.328	0.70
			IV	8.74	27.18	3.11		0.414	0.79
			V	8.93	28.19	3.16		0.153	0.78
Tryptophane, 14.5 grams	1.99	7	I			3.63			
			II	3.44	13.20	3.80		0.152	
			III	4.52	15.18	3.36	2.7	0.149	
			IV	5.12	15.94	3.11		0.294	
α -Hydroxyiso- valeric Acid, 15 grams		12	I			3.41			
			II	7.04	24.93	3.40		0.255	
			III	6.16	19.80	3.21	0.9	1.132	
			IV	7.22	20.72	2.97		0.346	

TABLE III

SUBSTANCE	WEIGHT OF SUBSTANCE	WEIGHT OF DOG	VOLUME PERFUSION FLUID	TIME OF PERFUSION	ACETO- ACETIC ACID FOUND
	<i>grams</i>	<i>kgm</i>	<i>cc</i>	<i>min</i>	<i>mgm</i>
Blank *		11	1100	50	51
Ornithine Carbonate	2 0	12	1100	45	42
Lysine Carbonate	2 0	15	1200	45	60
Lysine Carbonate	2 0	6	1070	50	67
Arginine Carbonate	3 5	13	1000	45	44
Histidine Carbonate*	2 5	12	1200	50	86
Proline	2 0	9	950	45	49
Di-iodotyrosine	2 0	7	1000	50	44
Tryptophane	1 5	13	1210	45	54

Average of many experiments

A NEW METHOD OF ISOLATING TRYPSIN

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INTRODUCTION

It has been pointed out by Robertson¹ that "if one drop of a saturated solution of safranin (Grubler) be added to from 5 to 10 cc of a neutral or very faintly alkaline 0.5 per cent solution of trypsin (Grubler) a light, flocculent, colored precipitate slowly appears on standing and gradually settles" Robertson assumes that this precipitate is a compound of trypsin and safranin and infers that trypsin, in faintly alkaline or neutral solutions, behaves like an acid and combines with the color-base safranin to form an insoluble salt

The present investigation was undertaken, in the first place, with a view of testing the accuracy of Robertson's assumption that the substance precipitated by safranin is actually trypsin, and, in the second place, the correctness of this assumption having been proven, to endeavor to utilize this precipitate for the isolation of the proteolytic agent from pancreas extracts and commercial preparations of "trypsin"

The proteolytic activity of the safranin precipitate

I have prepared the safranin precipitate from aqueous solutions of Grubler's and of Fairchild's trypsins and from aqueous extracts of sheep's pancreas and liver, prepared by grinding up the organs with sand and an equal weight of water and filtering. The precipitate was obtained by adding to these solutions three-eighths of their volume of an 0.8 per cent solution of Grubler's safranin.

The yield was always very small. Two and one-half grams of Grubler's trypsin dissolved in 100 cc of water yielded, on the aver-

¹ T. B. Robertson. *This Journal*, 11, p. 343, 1907

age, a precipitate weighing from 60 to 75 mgm when washed in alcohol and dried over sulphuric acid. Owing to its content of safranin the precipitate is of a deep red color and it is, moreover, almost insoluble in water. I have made many attempts to extract the safranin from the compound by repeatedly washing with alcohol. The compound would appear to be slightly soluble in alcohol, however, as the amount of material diminished progressively during this process. It proved, furthermore, impossible to extract the color from it, although the tint changed from red to purple. Prolonged extraction with alcohol also caused the precipitate to become very sticky and rendered it difficult or impossible to scrape it off the filter. I attempted to employ other solvents for extracting the safranin from the precipitate, namely, methyl alcohol, chloroform and benzine, but these were even less effective than ethyl alcohol and ether.

Despite its very slight solubility in water the safranin precipitate from Grubler's trypsin and pancreas extracts exerts a very energetic proteolytic action. The action of the precipitate from Fairchild's trypsin was only slightly inferior.

To 100-cc samples of a 2 per cent solution of casein in dilute KOH, neutral to phenolphthalein, I added 2 cc of water containing 20 mgm of the safranin precipitate from various sources suspended in it. For the purpose of comparison I added to a similar sample of casein solution a solution of 20 mgm of Grubler's trypsin in 2 cc of water. After stirring, these mixtures were allowed to stand for three hours at 35°C. I then determined the relative amounts of casein digested, employing Robertson's ² refractometric method and obtained the following results:

TABLE I.

			GRAMS OF CASEIN DIGESTED PER 100 CC OF DIGEST AFTER 3 HOURS STANDING AT 35 C
Safranin precipitate	(Grubler)		1.144
"	"	"	0.934
"	"	(Fairchild)	0.724
"	"	(Pancreas)	1.029
"	"	"	0.934
"	"	(Liver)	0.514
Trypsin (Grubler)			1.973

¹ T. B. Robertson. *This Journal*, xii, p. 23, 1912.

It will thus be seen that the proteolytic activity of these preparations was comparable with that of Grubler's trypsin, although they were nearly insoluble, while Grubler's trypsin is nearly completely soluble. We may infer, therefore, that if it were possible to free the preparation from safranin and so obtain it completely in solution its proteolytic activity would be far higher than that of Grubler's trypsin. Hence it is certain that trypsin is actually precipitated by safranin, and probable, also, that this precipitation separates the trypsin from the large proportion of inert impurities present in other preparations.

The presence of a large proportion of relatively inert substances in commercial preparations of trypsin

If we remove the substance precipitable by safranin by adding three-eighths of its volume of an 0.8 per cent solution of safranin to a 2.5 per cent solution of Grubler's trypsin and filtering, on adding several volumes of alcohol to the filtrate, we obtain a rather heavy, white and flocculent precipitate. On adding to this filtrate progressively increasing volumes of alcohol we obtain progressively increasing yields of precipitate until a maximum yield is obtained by the addition of from five to six volumes of absolute alcohol. This is illustrated by the following results.

TABLE II

25 cc filtrate plus	25 cc	absolute alcohol	no precipitate
" " " "	50 "	" "	trace
" " " "	75 "	" "	0.020 grams
" " " "	100 "	" "	0.150 "
" " " "	125 "	" "	0.225 "
" " " "	150 "	" "	0.255 "
" " " "	175 "	" "	0.225 "
" " " "	200 "	" "	0.265 "
" " " "	225 "	" "	0.265 "

The average yield of the alcohol precipitate was from 0.8 to 1.2 grams per 100 cc of the filtrate to which 600 cc of absolute alcohol were added, corresponding to a yield of from 1.1 to 1.6 grams from 2.5 grams of Grubler's trypsin. It is white in color or slightly pink owing to the presence of traces of safranin. It is readily soluble in water, but possesses extremely little proteolytic activity (see table III).

In the filtrate obtained after the removal of the safranin- and alcohol-precipitable substances from Grubler's trypsin colorless needles were deposited upon evaporation. These were probably crystals of magnesium sulphate.

An alcohol precipitate can be obtained by adding alcohol directly to a pancreas extract or to a solution of Grubler's trypsin. This precipitate is white in color and contains the active substance present in the safranin precipitate, as shown by its high proteolytic activity (see table III). Only a relatively small amount of the alcohol precipitate can be obtained from Fairchild's trypsin.

The precipitate which is obtained by adding alcohol directly to a solution of Grubler's trypsin is soluble in water. On adding safranin to this solution a precipitate is obtained.

After the removal of the safranin- and alcohol-precipitable substances from aqueous extract of sheep's pancreas, yet another substance can be precipitated by the addition of ether. This substance is soluble in alcohol and ether, but is insoluble in a mixture of the two in the rather definite proportion of six parts of alcohol to two of ether. It is soluble in mixtures of chloroform and ether and does not yield the biuret reaction. When dry it is white or faintly tinged with pink and slimy in consistency. This substance could not be obtained from Grubler's or Fairchild's trypsins.

The proteolytic activities of the alcohol and ether-alcohol precipitates were determined in a manner described above. In each case 20 mgm of the substance were dissolved in 2 cc of distilled water and added to 100 cc of a 2 per cent solution of casein. The amounts of casein digested were determined after three hours' digestion at 35°C. The results are shown in table III.

From these figures it is clear that as compared with the safranin precipitate or with the "direct" alcohol precipitate the alcohol precipitate which is obtained from Grubler's trypsin or from pancreas extract after removal of the safranin-precipitable substance possesses extremely little proteolytic action, no more, in fact, than might be attributable to contamination with a trace of the safranin-precipitable substance. This fact confirms the view that the substance precipitated by safranin is actually trypsin and also shows that Grubler's and Fairchild's trypsins contain a large proportion of inert substances. It is also clear that trypsin (combined, however, with safranin) can be prepared in a much purer condition,

TABLE III

		GRAMS OF CASEIN DIGESTED PER 100 CC OF DIGEST AFTER 3 HOURS STANDING AT 35 C	
(1)	Precipitate obtained by adding alcohol directly to Grubler's trypsin	0 618	grams
(2)	Precipitate obtained by adding alcohol directly to pancreas extract	1 447	"
(3)	Precipitate obtained by adding alcohol to the filtrate after removing the safranin-precipitable substance from Grubler's trypsin	0 105	"
(4)	Another precipitate of the same	0 211	"
(5)	" " " " "	0 092	"
(6)	" " " " "	0 198	"
(7)	Precipitate obtained by adding alcohol to the filtrate after removing the safranin-precipitable substance from pancreas extract	0 105	"
(8)	Another precipitate of the same	0 198	"
(9)	" " " " "	0 092	"
(10)	Ether-alcohol precipitate from pancreas extract (after removal of the safranin- and alcohol-precipitable substances)	0 000	"
(11)	Another precipitate of the same	0 105	"

in one operation, namely, by adding safranin to an aqueous pancreas extract and collecting the precipitate

Each of the fractions isolated, namely, the safranin, alcohol and ether-alcohol precipitates, were tested for lipolytic activity by employing triacetin as a substrate. None of them caused any splitting of this fat

SUMMARY

1 The substance, which is precipitated by the addition of safranin to aqueous solutions of Grubler's or Fairchild's trypsin or to aqueous extracts of pancreas, has a strong proteolytic action

2 This precipitate contains safranin and is very sparingly soluble in water. The author has not yet been able to extract the safranin from the compound or to render it more soluble in water

3 The safranin-precipitable substance having been removed from aqueous solutions of commercial trypsin or pancreas extracts, considerable quantities of substances remain in solution which are precipitable by alcohol (Grubler's and Fairchild's trypsin and pancreas extract) and by an alcohol-ether mixture of definite composition (pancreas extract). These substances are practically devoid of proteolytic activity

STUDIES ON THE CONDITIONS AFFECTING THE FORMATION AND EXCRETION OF FORMIC ACID

THE ESTIMATION OF FORMIC ACID IN URINE

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(Received for publication, March 10, 1913)

But little is known of the excretion of the volatile fatty acids in urine. Of their constant presence there can be no doubt, but knowledge as to their amount and the conditions affecting their formation and excretion is fragmentary. The reason for this is, no doubt, in part due to the inadequacy of existing methods for their determination and separation. For some time, we have been concerned with the problems of volatile fatty acid excretion and at present have occupied ourselves with devising a method for the estimation of the simplest of these acids, namely, formic acid. The fact that formic acid has characteristic reducing properties not shared by the other members of the group, tends to simplify the problem of its determination, but it required but little work to reach the conclusion that an accurate estimation of formic acid in a complex organic fluid, such as urine, is a very difficult matter. It is doubtless for this reason that no trustworthy estimation of formic acid in urines has been, as yet, recorded.

Hitherto, the methods made use of for the isolation of volatile fatty acids in urine have been based upon their preliminary separation by distillation with or without steam from the acidified urines. But at the outset, this process introduces most serious error. The prolonged action of mineral acids upon carbohydrate and other substances, invariably present to a greater or less extent in urines, results in volatile fatty acid production, especially formic acid. In the case of diabetic urines, enormous quantities of formic acid may be produced in this manner, owing to the well-known cleavage of glucose into laevulinic and formic acids. The errors arising from these causes may be diminished by using weaker acids for

liberating the volatile acids, by distilling under reduced pressure at lower temperatures and by avoiding undue concentration of the urine during distillation, but the method, in our hands at least, is fundamentally unsatisfactory

In connection with attempts to devise a better method, other sources of error were frequently encountered. One of these, if overlooked, is apt to result in gross inaccuracies. Thus, if a urine be rendered alkaline with carbonate of soda and evaporated on the water bath in order to concentrate the urine without loss of volatile fatty acids, it is found that the concentrated urine, especially when carbohydrates are present, may contain several times as much volatile fatty acids after concentration as before. Thus, for example, diabetic urines from dogs which actually contained from 20-50 mgms of formic acid in the twenty-four hours' collection, after evaporation to one-half the original volume on the water bath with a slight excess of carbonate of soda, were found to contain from 80 to over 1300 mgms. The same change is observed, but to a much lesser extent, in urines free from excess of carbohydrates.

An additional difficulty in arriving at a correct estimate of formic acid in urine was found in the curious variations shown in the formic acid contents of urines on standing. Frequently an increase is observed, but often there is a decrease even when the urine is apparently in good condition. Strong antiseptics are not admissible, but even when the urine is preserved at low temperatures in the presence of toluene, it is important to commence the analysis as soon as possible. Urines showing any bacterial contamination must of course be rejected and, since organisms may act very rapidly, especially in urines containing sugar, it is important that samples should be protected from decomposition as soon as possible after voiding. Even slight fecal contamination at once invalidates the results.

The foregoing considerations led us to the belief that in order to attempt the estimation of formic acid and other volatile acids in urines, it would be necessary to devise a method which would remove the acids from the main bulk of urinary constituents with a minimum of chemical manipulation. With this end in view, we have worked out a procedure which, while far from ideal, does, we believe, offer substantial advantage over current methods.

The method is based upon the observation that it is perfectly feasible, by means of ether, to extract at low temperatures all of the volatile fatty acids from acidified urine or other dilute aqueous solutions¹. The extraction is performed in any of the usual forms of apparatus arranged for continuous extraction, and in order to prevent the volatile acids, once extracted from the aqueous solution, from returning, we place an excess of carbonate of soda solution in the ether reservoir, thus promptly neutralizing and fixing the volatile acids as fast as they are extracted. By this means the volatile acids are obtained in alkaline solution free from the main bulk of urinary constituents, carbohydrates, purine bodies, amino-acids, etc., which interfere with an accurate estimation of formic or other volatile acids.

On acidifying the sodium carbonate solution with phosphoric acid and distilling in steam, the distillate contains all of the volatile fatty acids and may be used for the determination of formic acid by appropriate methods. The method based upon the reduction of mercuric chloride to calomel has been found by us to be the most satisfactory. The details of the method are described later.

The extraction of formic acid from aqueous solutions by ether

Preliminary experiments were made to determine the conditions governing the extraction of formic acid by ether. About half a gram of formic acid in 130 cc. of water was extracted with ether in a continuous extractor for varying lengths of time. Excess of alkali was placed in the ether reservoir. In order to determine the amount of formic acid still remaining in the aqueous solution after varying times of extraction, 5 cc. or more of the solution were removed at intervals and titrated against decinormal baryta.

In experiments I and II, the return flow of ether was fairly rapid, though not a continuous stream, but in experiment III, the flow of ether was much slower. The results show that formic acid

¹ The fact that formic acid can be extracted from aqueous solutions with ether, appears not to be generally known. Curtius and Franzen, in order to demonstrate the presence of formaldehyde in plants, oxidized the aldehydes to acids and extracted with ether to remove the higher acids. This procedure must lead to some loss of formic acid. (*Ber. d. deutsch. chem. Gesellsch.*, xlv, p. 1715, 1912.)

EXPERIMENT	TIME OF EXTRACTION	TITRATION OF 5 CC AQUEOUS SOLUTION WITH $\frac{N}{16}$ BARYTA	PERCENTAGE OF FORMIC ACID EXTRACTED BY ETHER
I	Before extraction	4 20	0
	After 1 hour	1 65	61
	After 2 hours	0 35	92
	After 3 hours	0 05	99
	After 4 hours	Less than 1 drop	100
II	Before extraction	4 20	0
	After 1 hour	1 20	71
	After 2 hours	0 40	90
	After 3 hours	0 15	96
	After 4 hours	Less than 1 drop	100
III	Before extraction	5 10	0
	After 1 hour	3 50	31
	After 3 hours	0 40	92
	After 4 hours	0 15	97
	After 6 hours	Less than 1 drop	100

is comparatively rapidly and completely removed from its aqueous solution when extracted with ether under the existing conditions. After four hours' extraction almost the whole of the formic acid had been removed. In our subsequent determinations of formic acid, we allowed the extraction to continue for twelve hours, thus allowing a considerable margin of safety.

We have found that in order to recover the formic acid after extraction by ether and fixation with alkali, it is most convenient to separate the aqueous portion from the ether, acidify strongly with phosphoric acid and distil in a current of steam. The dilute formic acid solution may then be neutralized with a distinct excess of caustic soda, evaporated on the water bath and is then, after neutralization with acetic acid, ready for gravimetric estimation by means of the mercuric chloride method. That the procedure when carefully carried out does not involve loss was shown by taking 0.0964 gram of formic acid and recovering 0.0963, 0.0980, 0.0968 gram in three consecutive experiments.

Conditions affecting the determination of formic acid by mercuric chloride

The determination of formic acid, based upon its reducing action upon mercuric chloride with formation of calomel, has been studied by Scala,² Lieben,³ Leys⁴ and others. As there appeared to be a certain lack of agreement as to the most favorable conditions, we have reinvestigated some of the principal points.

1 *Time and mode of heating* The reaction between formic acid and mercuric chloride is a slow one and needs prolonged heating for its completion. Since heavy precipitates of calomel are apt to result in violent "bumping," if the solution be boiled, we prefer to immerse the flasks provided with an air-cooled condenser tube, in a bath of boiling water. The calomel was collected on weighed Gooch crucibles, washed and dried in the water-oven. The subjoined results show that it is necessary to heat for at least six hours.

SERIES	TIME OF HEATING	MERCURIC CHLORIDE USED	CALOMEL FORMED	FORMIC ACID PRESENT	FORMIC ACID FOUND	DIFFERENCE
	hours	grams	gram	gram	gram	gram
I	1	6.6	0.9211	0.0957	0.0900	-0.0057
	3	6.6	0.9556	0.0957	0.0933	-0.0014
	4	6.6	0.9719	0.0957	0.0949	-0.0008
	6	6.6	0.9802	0.0957	0.0957	
II	1.5	4.9	0.4041	0.0483	0.0394	-0.0089
	2.5	4.9	0.4481	0.0483	0.0437	-0.0046
	3.5	4.9	0.4721	0.0483	0.0461	-0.0022
	4.5	4.9	0.4808	0.0483	0.0470	-0.0013
	5.5	4.9	0.4863	0.0483	0.0475	-0.0008
	6.5	4.9	0.4935	0.0483	0.0482	-0.0001
III	5	4.9	0.4935	0.0483	0.0482	-0.0001
	6	4.9	0.4936	0.0483	0.0482	-0.0001
	7	4.9	0.4958	0.0483	0.0484	+0.0001
	8	4.9	0.4953	0.0483	0.0484	+0.0001
	9	4.9	0.4955	0.0483	0.0484	+0.0001
	10	4.9	0.4979	0.0483	0.0486	+0.0003

² Scala *Gaz chim ital*, xxvi, p 394, 1890

³ Ad Lieben *Monatsh f Chem*, xiv, p 753, 1893

⁴ A Leys *Bull de la soc chim de Paris*, (3), xix, p 472, 1898

2 *Amount of mercuric chloride* It is well recognized that, in order to obtain accurate results, the presence of a considerable excess of mercuric chloride is necessary. Our experiments (see following table) show that under the conditions of heating chosen by us it is essential to have at least six to eight times the amount theoretically necessary. In practice it is a good plan to use an amount of mercuric chloride equivalent to about one hundred times the weight of formic acid anticipated.

SERIES	TIME OF HEATING	MERCURIC CHLORIDE USED	CALOMEL FORMED	FORMIC ACID PRESENT	FORMIC ACID FOUND	DIFFERENCE
	hours		gram	gram	gram	gram
I	6	2 × theor amt	0.8789	0.0957	0.0858	-0.0099
	6	4 × theor amt	0.9635	0.0957	0.0940	-0.0017
	6	6 × theor amt	0.9802	0.0957	0.0957	
II	6	3 × theor amt	0.4021	0.0477	0.0392	-0.0035
	6	4 × theor amt	0.4316	0.0477	0.0421	-0.0056
	6	5 × theor amt	0.4566	0.0477	0.0416	-0.0031
	6	6 × theor amt	0.4639	0.0477	0.0452	-0.0025
	6	7 × theor amt	0.4810	0.0477	0.0469	-0.0008
	6	8 × theor amt	0.4797	0.0477	0.0468	-0.0009

3 *Effect of sodium acetate and of acetic acid* Since, in the course of estimating the formic acid in urine, the distillates were made alkaline with caustic soda and then concentrated and it was proposed to acidify with acetic acid before heating with mercuric chloride, it was necessary to investigate the effect of sodium acetate and of acetic acid. The subjoined results show that under the existing conditions an excess of acetic acid up to at least 30 cc of decinormal strength was without effect. Large amounts of sodium acetate tend to give high results probably due to the precipitation of a little mercurous acetate. Such amounts as occur in the course of urinary analysis would be without effect.

FORMIC ACID TAKEN	% ACETIC ACID ADDED	CRYST SODIUM ACETATE ADDED	CALOMEL FORMED	FORMIC ACID FOUND	DIFFERENCE
gram	cc	grams	gram	gram	gram
0.0477			0.4846	0.0473	-0.0004
0.0477	10		0.4841	0.0472	-0.0005
0.0477	30		0.4868	0.0475	-0.0002
0.0477	10	2	0.4992	0.0487	+0.0010
0.0477		5	0.5008	0.0488	+0.0011
0.0477		10	0.5107	0.0493	+0.0021

The estimation of formic acid in urine

The procedure that has been found most satisfactory is as follows. As large a volume of fresh urine as may be conveniently handled is precipitated with solid ammonium sulphate, using about 20 grams for each 100 cc of urine. Aliquot portions of the filtrate are acidified with phosphoric acid and extracted for twelve hours with a rapid flow of ether. We have commonly used 250 cc of urine and 10 cc of 50 per cent phosphoric acid. It is well to use ether previously shaken with caustic soda solution. The flask in which the ether is boiled contains about 20 cc of 5 per cent sodium carbonate solution.

At the close of the extraction, the contents of the flask are transferred to a separatory funnel and the alkaline solution is allowed to flow into a flask suitable for steam distillation. The ether is washed twice with a little water and the washings added to the flask. The whole is then acidified with phosphoric acid and the volatile acids recovered by distilling in a rapid current of steam. Ordinarily, it suffices to collect a liter of distillate. If necessary, the distillate is filtered through a wet filter paper in order to remove traces of higher fatty acids. It is convenient to determine the total acidity by titrating an aliquot part of the distillate (100 cc) with decinormal baryta and phenolphthalein. Often considerable variation in the acidity, as determined by titration of duplicates, may occur, owing to the presence of carbon dioxide in varying quantity. The remainder (900 cc) is then made distinctly alkaline by adding caustic soda solution and concentrated on the water bath to about 50 cc. It is convenient to neutralize with alkali of known strength and to add a definite excess—say 20 cc of decinormal solution—as in this case the subsequent acidification is most readily managed. Dilute acetic acid is now added, after cooling, in amount slightly more than that necessary to neutralize the excess of caustic soda, so as to render the solution distinctly acid to litmus. After filtering into an Erlenmeyer flask, excess of mercuric chloride is added. For each cubic centimeter of total acidity measured with decinormal alkali, it is well to use 5 cubic centimeters of saturated mercuric chloride solution. The addition of the mercuric chloride is occasionally followed by an immediate trifling turbidity which cannot be easily removed by filtra-

tion The origin of this turbidity is unknown but it is not due to lower fatty acids, lactic benzoic, phenylacetic or hydroxyphenylacetic acids, and since the relative weight of calomel obtained from formic acid is so large, it does not introduce a significant error

The flask is now provided with a short air-cooled condenser tube and heated for at least six hours by immersion in a boiling water bath After cooling, the precipitate is filtered off on a weighed Gooch crucible and washed with 100 cubic centimeters of cold 5 per cent hydrochloric acid, then successively with water, alcohol and ether Finally it is dried for about two hours in the water-oven and weighed (1 gram calomel = 0.0977 gram formic acid) The object of washing with the hydrochloric acid is to remove some slight impurities which are apt to occur in the precipitates obtained from urines, while the calomel is substantially unattacked, provided the hydrochloric acid is not used hot It is advisable to make blank tests on the reagents as they will usually be found to give 5-10 mgms of precipitate equivalent to 0.5-1 mgm of formic acid

Oxalic, lactic and crotonic acids do not act as disturbing factors in this estimation of formic acid

Formic acid as a product of intermediary metabolism

The constant presence of formic acid in urines and the comparative ease with which it undergoes oxidation in the animal body, makes it appear probable that it is an important product of intermediary metabolism, but there is an almost entire lack of information concerning the origin of the acid

Pohl showed that an increased formic acid excretion followed administration of formaldehyde, methylamine, methyl alcohol and some derivatives of these substances, but these results have little bearing on the processes of normal metabolism More recently Steppuhn and Schellbach⁵ reported an increase in formic acid output following glucose administration by mouth and they believe that its formation may be demonstrated when liver tissue undergoes autolysis in the presence of glucose The risk of bacterial decomposition of sugar with production of formic acid in these experiments would appear to be great and in addition we are inclined to believe that the method of analysis employed was not adequate to

⁵ *Zeitschr f physiol Chem*, lxxx, p 274, 1912

the difficult task of estimating formic acid in the presence of much glucose

Our experiments are concerned with the following points

I Formic acid in normal human urines

II The influence of starvation, and of carbohydrates and proteins on the formic acid excretion

III The relation of higher fatty acids and other substances to formic acid production

I *Formic acid in normal urines* Twelve analyses of normal human urines, from individuals on mixed diets, gave results varying from 29.9 to 118.6 mgms in the twenty-four hours with an average excretion of 60.3. There was no obvious relation between weight, urine volume or nitrogen excretion. These data are therefore omitted.

On increasing the consumption of carbohydrates, the formic acid excretion shows a tendency to increase (35.0–175 mgms per 24 hours. Average, 96 mgms). The effect of excessive protein is similar but less marked (34.6–99 mgms per 24 hours. Average, 67 mgms). These effects are seen more clearly in the experiments on dogs.

II *Effect of starvation, carbohydrates and proteins* The excretion of formic acid in animals rapidly falls on starvation to about one-third the normal amount. The average result of six experiments on dogs weighing 10–12 kgms is 8.9 mgms per 24 hours. On administering carbohydrates (potatoes) to these animals a marked increase in formic acid is observed. The average excretion on a potato diet was 51 mgms. On feeding meat freely, a very definite increase over the output during starvation is observed, but the effect is somewhat less than that observed with carbohydrates. The average output on a meat diet for the same animals was 27 mgms.

Since it is known that many intestinal microorganisms may not only ferment but also produce formic acid from both carbohydrates and proteins, it was conceivable that these increases in formic acid excretions were due to bacterial activity. That this explanation does not account for the rise was shown by the fact that a marked increase in formic acid output was found to follow intravenous injections of glucose given to fasting dogs. It seems likely therefore that formic acid is an intermediate product of the catabolism

of carbohydrates and proteins. The relatively small amount of the acid actually excreted is due in part, no doubt, to the fact that it undergoes further oxidation in the body with ease. It is at present impossible to form an estimate of the amount actually produced.

An attempt was made to gain some insight into the protein constituents which were capable of yielding formic acid by administering to cats and rabbits certain amino-acids and allied substances (table III). Glycine, glycollic acid, alanine, lactic acid and aspartic acid led to no definite rise in formic acid excretion, but two experiments, in which relatively large amounts of histidine hydrochloride were given to cats, resulted in a very definite increase in formic acid output. It is noteworthy, too, that phlorhizin glycosuria induced in starving dogs, accompanied by a large increase in protein catabolism, is followed by a rise in formic acid output.

III *The relation of formic acid to higher fatty acids*. In a preliminary note⁴ published by two of us (H D D and A J W) it was stated that "the administration of the sodium salts of fatty acids may result in the excretion of from ten to thirty times the normal amount of formic acid." This statement was made on the basis of more than fifteen separate experiments, but as the result of a much larger number, carried out subsequently, we are forced to the conclusion that we largely overestimated the increase and we therefore wish to correct our earlier statement. The later experiments show that while an increase in formic acid output does commonly follow administration of the sodium salts of fatty acids, it seldom amounts to more than three to four times the normal output. In our earlier experiments we were not aware of many of the sources of error in formic acid analyses referred to at the commencement of this paper.

A few of our later experiments were recorded in table III. In order to further test the possibility of formic acid arising from the oxidation of higher fatty acids in the body, experiments were made to determine the formic acid in blood to which sodium acetate and propionate had been added before and after perfusion of the surviving liver of dogs. Our experiments gave negative results and therefore, it does not in

In conclusion, reference may be made to the possible production of formic acid from caffeine and theobromine when these substances undergo demethylation in the animal body. Preliminary experiments have given encouraging results and the question will be submitted to a careful examination.

SUMMARY

An improved method for the estimation of formic acid is described.

The formic acid present in the urine is in part of endogenous origin. The excretion of formic acid is greatly reduced during starvation but is largely increased when carbohydrates are given, either by mouth or when glucose is given intravenously. Protein feeding is followed by a similar but smaller increase in formic acid output. Formic acid appears to be a product of the intermediary metabolism of carbohydrates and proteins.

The effect of a number of other substances on formic acid excretion was investigated, including amino, hydroxy and saturated fatty acids.

TABLE I¹

Effect of fasting, carbohydrates and proteins on formic acid excretion

EXPERIMENT	WEIGHT OF DOG	FORMIC ACID	TOTAL NITROGEN	DIET	
	<i>kgm</i>	<i>mgm</i>	<i>grams</i>		
I	11	6 6	2 5	Fasting Potatoes and gravy Meat	Average for 3 days
		60 0	4 7		Average for 2 days
					Average for 2 days
		27 5	20 5		
II	13	8 3	3 9	Fasting Meat Potatoes Fasting	Average for 3 days
		30 0	31 5		Average for 4 days
		62 0	3 6		Average for 4 days
		44 0	6 7		Phlorhizin glycosuria
III	12 7	9 5	3 0	Fasting Potatoes	Average for 2 days
		32 0	2 1		

¹ All figures refer to twenty-four-hour periods.

TABLE II

Effect of intravenous injections of glucose on formic acid excretion

EXPERIMENT	WEIGHT OF DOG	FORMIC ACID	TOTAL NITROGEN	CONDITIONS
	kgm	mgm	grams	
I	11	14 1 69 0	4 5 5 3	Average for 2 days after 3 days fasting 200 grams glucose given intravenously 97 6 grams excreted unchanged
II	12 7	9 5 23 4	3 0 3 5	Average for 2 days 80 grams glucose given intravenously 33 grams excreted unchanged
III	8 9	5 7 45 8	1 9 3 0	Fasting 71 grams glucose given intravenously 28 5 grams excreted unchanged
IV	10	8 2 47 8	2 6 3 0	Fasting 100 grams glucose given intravenously 37 grams excreted unchanged

TABLE III

Effect of various substances on formic acid excretion

ANIMAL	WEIGHT	FORMIC ACID 24 HOURS	TOTAL NITROGEN	SUBSTANCES GIVEN*
	kgm	mgm	grams	
{ Cats	2-3 5	2 0-5 6		Fasting Average of 5 experiments
{ Cats	2-3 5	6 3-9 4		Meat diet Average of 3 experiments
Cat		5 9	1 88	Sodium chloride 100 cc 0 85 per cent intravenously
Cats	2-3 5	2 1-7 9		Sodium bicarbonate 3-5 grams intravenously Average of 5 experiments
{ Rabbit	1 9	3 4	1 7	
{ Rabbit	1 9	9 1	0 8	Acetic acid, 2 grams by mouth

* All acids in form of sodium salts
Intravenous injections usually measured 80-150 cc and lasted one to four hours.

TABLE III—Continued

ANIMAL	WEIGHT	FORMIC ACID 24 HOURS	TOTAL NITROGEN	SUBSTANCES GIVEN*
	<i>kgm</i>	<i>mgm</i>	<i>grams</i>	
Cats	2 5-3 0	13 0-44 5		Acetic acid, 3-4 5 grams intra- travenously
Cat	3 0	23 0	2 34	Propionic acid, 5 grams intra- travenously
Cat	3 0	19 0		Propionic acid, 7 grams intra- travenously
{ Rabbit	1 2	5 2	0 8	
{ Rabbit	1 2	10 1	1 3	Butyric acid, 2 grams by mouth
Cat	3 5	29 8	4 50	Butyric acid, 5 grams intra- travenously
Cat		13 7	0 59	Glycollic acid, 4 grams intra- travenously
{ Rabbit	1 8	7 1	1 9	
{ Rabbit	1 8	7 9	1 5	Lactic acid, 2 5 grams by mouth
{ Rabbit	1 2	3 4	1 7	
{ Rabbit	1 2	3 7	1 1	Acetoacetic acid, 2 5 grams by mouth
Cat	3 5	11 7	5 22	Glycocoll, 5 grams intraven- ously
Cat	2 7	9 3	4 5	Alanine, 6 grams intraven- ously
Cat	3 0	12 3	5 04	Aspartic acid, 6 grams intra- travenously
Cat	2 8	27 0	2 73	Histidine carbonate, 2 grams intravenously
{ Cat	2 8	6 5		
{ Cat	2 8	42 4		Histidine carbonate, 3 grams intravenously
Cat	3 5	82 0		Histidine hydrochloride, 3 5 grams subcutaneously
Cat	3 1	430 0	3 03	Methyl alcohol, 10 grams intra- travenously
Cat	3 1	8 7	1 89	Ethyl alcohol, 20 grams intra- travenously

All acids in form of sodium salts
Intravenous injections usually measured 80-150 cc and lasted one to four hours

THE RELATIVE INFLUENCE OF WEAK AND STRONG BASES UPON THE RATE OF OXIDATIONS IN THE UNFERTILIZED EGG OF THE SEA URCHIN

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1 About six years ago it was shown by Loeb that bases (NaOH, KOH) can induce artificial parthenogenesis in the eggs of sea urchins and annelids¹ This action of the bases was suppressed or retarded when the oxidations in the egg were suppressed or retarded by the withdrawal of oxygen from the alkaline solution or by the addition of a small amount of KCN² He therefore concluded that the bases induced artificial parthenogenesis through an acceleration of the rate of oxidations in the egg Last summer the same author showed that the weak base NH_4OH is much more efficient for the causation of artificial parthenogenesis than the strong bases NaOH, KOH or tetraethylammoniumhydroxide³ This he explained on the basis of the fact found by O Warburg,⁴ and extended by Harvey,⁵ that the weak bases diffuse more readily into the egg while the strong bases do not This behavior was an analogue to the fact found by Loeb in 1905 that weak acids like CO_2 or the monobasic fatty acids induce membrane formation and development readily in the unfertilized egg of the sea urchin, while the strong acids like HCl or oxalic acid are very ineffective⁶ This fact he explained on the assumption that the weak acids diffuse readily into the egg while the strong acids do not While the bases

¹ Loeb *Pflüger's Archiv*, cxviii, p 572, 1907

² Loeb *ibid*, cxviii, p 30, 1907

³ Loeb *Journ of Exp Zoology*, xii, p 577, 1912

⁴ Warburg *Zeitschr f physiol Chem*, lxvi, p 305, 1910

⁵ Harvey *Journ of Exp Zoology*, x, p 507, 1911

⁶ Loeb *Univ of Calif Publ Physiol*, ii, p 113, 1905, *Biochem Zeitschr*, xv, p 254, 1909

only acted in the presence of oxygen, the action of the acids was independent of oxidations in the egg

2 More recently Loeb extended his investigations on the relative efficiency of weak and strong bases for artificial parthenogenesis to a larger number of bases. The egg used was that of an annelid, *Polynoe*. It was found that the bases, in regard to their efficiency for this purpose, may be divided into three groups. The most efficient were the amines of which benzyl, butyl, ethyl and methylamine were tried. Benzyl and butylamine were possibly a little more efficient than ethyl and methylamine. Next in efficiency were NH_4OH and trimethylamine. The least efficient were the strong bases NaOH and tetraethylammoniumhydroxide.

The relative efficiency of these bases for the causation of artificial parthenogenesis was tested in this way that the unfertilized eggs of *Polynoe* were put into solutions containing the same molecular concentration of these various bases. The reciprocal value of the time required for the various bases to cause development was the measure of their relative efficiency. The simple amines acted most quickly, then followed NH_4OH and trimethylamine, the strong bases required more time than either of the two other groups of bases.

3 These experiences suggested an investigation of the influence of the various bases upon the rate of oxidations in the unfertilized egg, to find out whether the weaker bases raised the rate of oxidations more than the stronger bases.

The experiments were carried out on the unfertilized egg of *Strongylocentrotus purpuratus* in Pacific Grove, California. The oxygen consumption was determined according to Winkler's methods. The experiments were made in this way that the oxygen consumption for the same lot of eggs was first determined in a neutral solution and then for the same length of time and the same temperature in an alkaline solution. The experiments were made in a half gram molecular mixture of $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ in that proportion in which these three salts are contained in the sea water. The reader who is interested in the details of the method may be referred to our former publications or to those of Warburg.⁷

⁷ Loeb and Wasteneys *Biochem Zeitschr*, xxviii, p. 340, 1910, Warburg
loc. cit.

We first give the results of a series of experiments in which the relative influence of various bases was compared. The time of exposure was one hour and twenty-five minutes, the temperature, 18°C. The concentration of the bases chosen was that found most effective in Loeb's previous experiments on artificial parthenogenesis. The oxygen consumption was first measured in a neutral solution and then for the same eggs in the alkaline solution in which 0.3 cc. of $\frac{N}{10}$ of the various bases was added to 50 cc. of the solution.

TABLE I

NUMBER OF EXPERIMENT	NATURE OF SOLUTION	OXYGEN CONSUMED	ACCELERATION OF RATE OF OXIDATION BY THE BASE
		mgm	
I	{ Neutral 50 cc neutral + 0.3 cc $\frac{N}{10}$ NaOH	0.28 0.40	1.43
II	{ Neutral 50 cc neutral + 0.3 cc $\frac{N}{10}$ tetra-ethylammoniumhydroxide	0.15 0.22	1.50
III	{ Neutral 50 cc neutral + 0.3 cc $\frac{N}{10}$ NH ₄ OH	0.30 0.81	2.70
IV	{ Neutral 50 cc neutral + 0.3 cc $\frac{N}{10}$ trimethyl-amine	0.40 1.19	3.00
V	{ Neutral 50 cc neutral + 0.3 cc $\frac{N}{10}$ methyl-amine	0.25 1.18	4.70
VI	{ Neutral 50 cc neutral + 0.3 cc $\frac{N}{10}$ ethylamine	0.28 1.35	4.80
VII	{ Neutral 50 cc neutral + 0.3 cc $\frac{N}{10}$ butyl-amine	0.32 1.23	3.80
VIII	{ Neutral 50 cc neutral + 0.3 cc $\frac{N}{10}$ benzyl-amine	0.22 1.30	5.90

These experiments, which were repeated with the same result, show clearly that the relative efficiency of the bases for inducing artificial parthenogenesis in the unfertilized eggs of *Polynoe* and

the sea urchin runs parallel with their accelerating influence upon the rate of oxidations in the unfertilized egg of the sea urchin. Incidentally it may be stated that NaHCO_3 does not accelerate the rate of oxidations in the unfertilized egg nor does it cause artificial parthenogenesis.

4 We compared next the relative effect of various concentrations of NaOH and NH_4OH upon the rate of oxidations in the unfertilized sea urchin egg, during one hour. We will state only the coefficient of the rate of oxidation in the various solutions, calling the rate in the neutral solution 1.

TABLE II

AMOUNT OF BASE ADDED TO 50 cc $\frac{N}{2}$ ($\text{NaCl} + \text{KCl} + \text{CaCl}_2$)	COEFFICIENT OF ACCELERATION OF OXIDATIONS IN	
	NaOH	NH_4OH
0.2 cc $\frac{N}{10}$	1.35	1.29
0.5 cc $\frac{N}{10}$	1.87	5.00
0.8 cc $\frac{N}{10}$	1.74	5.94
1.0 cc $\frac{N}{10}$	2.06	6.10
1.4 cc $\frac{N}{10}$	2.32	6.40
2.0 cc $\frac{N}{10}$	3.42	6.23
2.5 cc $\frac{N}{10}$	4.57	5.70
3.0 cc $\frac{N}{10}$	7.60	6.00

The reader will notice the striking difference in the behavior of NH_4OH and NaOH . Very low concentrations of NH_4OH (0.5 cc per 50 cc of solution) raise the rate of oxidations in the fertilized egg almost to the maximal height, and a further rise in the concentration has only a slight effect upon the rate of oxidation. Low concentrations of NaOH raise the rate of oxidation only little and its efficiency rises steadily with an increase in its concentration. We could not go beyond the concentrations used in this experiment since the addition of 3 cc of $\frac{N}{10}$ NaOH to 50 cc of $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ leads already to a cytolysis of the eggs.

It is also of interest to point out that in the eggs of *Strongylocentrotus purpuratus* fertilization by sperm raises the rate of oxidation to about six times that in the unfertilized eggs. This seems to indicate that with NH_4OH it is not possible to raise the rate of oxidations in the unfertilized egg beyond the limit to which it can be raised by the fertilization with sperm. It is not possible to decide whether the same holds true for NaOH .

The fact that NH_4OH reaches its maximum effect at so low a concentration is not confined to NH_4OH but is also shared by the amines, as the following table shows NH_4OH and ethylamine were compared

TABLE III

AMOUNT OF BASE ADDED TO 50 cc $\frac{N}{T}$ ($\text{NaCl} + \text{KCl} + \text{CaCl}_2$)	COEFFICIENT OF ACCELERATION OF OXIDATIONS IN	
	NH_4OH	Ethylamine
0 1 cc $\frac{N}{10}$	1 9	1 4
0 2 cc $\frac{N}{10}$	2 9	3 0
0 4 cc $\frac{N}{10}$	3 4	4 3
0 8 cc $\frac{N}{10}$	3 9	4 2

Ethylamine reaches its maximal efficiency at the concentration of 0 4 cc of $\frac{N}{10}$ base to 50 cc of the neutral liquid, and for NH_4OH the limit is nearly at the same point as in our previous experiment

5 It seems natural to connect this difference in the behavior of NaOH and NH_4OH with the difference in the rate of their diffusion into the unfertilized egg. If the rate of diffusion of NaOH is extremely slow and that of NH_4OH fast, it is natural that the maximal rate of oxidation should be reached with a lower concentration of NH_4OH than of NaOH . We determined the consumption of oxygen for the same lot of eggs for eight consecutive hours in 50 cc of sea water + 1 0 cc of $\frac{N}{10}$ NaOH . The following table gives the result

TABLE IV

Consumption of oxygen at 18° in 50 cc of normal sea water + 1 0 cc of $\frac{N}{10}$ NaOH in eight consecutive hours

	OXYGEN CONSUMED	COEFFICIENT OF OXIDATION
	mgm	
1st hour	0 24	1 00
2d hour	0 38	1 57
3d hour	0 45	1 87
4th hour	0 50	2 08
5th hour	0 58	2 42
6th hour	0 72	3 00
7th hour	0 92	3 84
8th hour	0 95	3 96

This table shows that the longer the NaOH acts upon the egg the higher the amount of oxygen becomes which is consumed per

hour This would agree with the assumption that the NaOH diffuses slowly into the egg and that the increase in the rate of oxidations in the unfertilized egg is determined by the amount of base which has diffused into the egg

6 It was to be expected that since NH_4OH is very soluble in the egg, *i e*, diffuses rapidly into the egg, its maximum effect would be reached during the first hour This was found to be true, as the following table shows

TABLE V

Consumption of oxygen by unfertilized eggs at 18° in 50 cc of normal sea water + 0.8 cc of $\frac{N}{10}$ NH_4OH

		OXYGEN CONSUMED	COEFFICIENT OF OXIDATION
		<i>mgm</i>	
Normal sea water		0.15	1.0
50 cc sea water + 0.8 cc $\frac{N}{10}$ NH_4OH	1st hour	0.99	6.7
50 cc sea water + 0.8 cc $\frac{N}{10}$ NH_4OH	2d hour	1.03	6.9
50 cc sea water + 0.8 cc $\frac{N}{10}$ NH_4OH	3d hour	0.87	5.8
50 cc sea water + 0.8 cc $\frac{N}{10}$ NH_4OH	4th hour	0.86	5.7
50 cc sea water + 0.8 cc $\frac{N}{10}$ NH_4OH	5th hour	0.83	5.5

7 We intend to investigate in future experiments whether these effects of bases upon the rate of oxidations in the unfertilized eggs are irreversible, *i e*, will continue if the eggs are put into normal sea water after the treatment with alkali But, we have an experiment which possibly serves the same purpose We measured the amount of oxygen consumed in one hour by the eggs mentioned in the last table in the same solution sixteen and twenty-four hours, respectively, after the experiment In the meantime the eggs had been kept at a low temperature in normal sea water The rate of oxidation after sixteen or twenty-four hours was practically the same as in the second hour

8 These experiments prove two facts, first, that the weaker bases increase the rate of oxidations in the unfertilized egg more than the stronger bases, and second, that this difference is due to the fact that the weaker bases diffuse more rapidly into the egg than the strong bases

The connection between the oxidative action of bases and artificial parthenogenesis lies in the fact that the essential factor in

artificial parthenogenesis is an alteration of the surface or cortical layer of the egg which results in a membrane formation. Loeb has shown in former experiments that bases cause the swelling and liquefaction of the gelatinous mass (the so-called chorion) which surrounds the immature egg of a mollusc, *Lotha*, and that this action of bases is inhibited by lack of oxygen and by the addition of KCN.⁸ This year the same author convinced himself that weak bases like the amines and NH_4OH bring about the dissolution of the chorion much more rapidly than the strong bases NaOH and tetraethylammoniumhydroxide. It is possible that the induction of artificial parthenogenesis in the sea urchin egg by bases depends upon the occurrence of a similar process in the cortical layer of this egg.⁹ We may imagine that they act by accelerating the rate of oxidation of a substance (existing in the cortical layer of the egg?) whereby the membrane formation and consequently the development of the egg is induced.

SUMMARY

The paper shows that the weak bases which are more efficient in causing artificial parthenogenesis are also more efficient in raising the rate of oxidations in the unfertilized egg. This lends further support to the view expressed by Loeb that the bases cause artificial parthenogenesis through an acceleration of the rate of oxidations.

The experiments were carried on at the Herzstein Laboratory in Monterey, California, and the authors express their thanks to Drs Robertson, Maxwell and Moore for their kind hospitality.

⁸ Loeb *Univ. of Calif. Publ. Physiol.*, III, p. 1, 1905.

⁹ Loeb *Die chemische Entwicklungserregung des tierischen Eies*, Berlin, 1909, p. 181.

FURTHER METABOLISM EXPERIMENTS UPON PARATHYROIDECTOMIZED DOGS

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In a previous paper,¹ the author described a series of metabolism experiments upon parathyroidectomized dogs. The most striking change in the metabolism observed after removal of the parathyroids was the very marked diminution in the excretion of phosphorus in the urine. This was not accompanied by an increase in the elimination of phosphorus in the feces. Apparently there was a retention of phosphorus in the body.

One of the questions that then arose was: Is this retention of phosphorus a primary metabolic disturbance or is it secondary to a retention of sodium or of potassium or of both? The experiments here reported were designed to answer this question. The results leave no doubt that the retention of phosphorus is primary. In none of the experiments was there any indication of a retention of sodium or of potassium preceding that of phosphorus. In three, the fall in the phosphorus elimination occurred a day before the excretion of sodium and potassium was affected. In the other two, the excretion of all three substances was diminished on the day of the operation.

The significance of these experiments is not altogether clear. It may be that, after parathyroidectomy, the ratio between the concentrations of calcium and of sodium and potassium in the body-fluids is altered not so much by loss of calcium as by retention of sodium and potassium. A few analyses of serum indicate that this is the case. Whether or not such a change can be regarded

¹ Greenwald *Amer Journ of Physiol* xxviii, p 103, 1911

as the cause of tetany is, of course, questionable. However, since these changes in the excretion of phosphorus, sodium and potassium are so marked, it seemed desirable that the content of these substances in the tissues be investigated. Some work has already been done on the amount and nature of the phosphorus compounds of the blood and serum of normal and parathyroidectomized dogs. An account of these experiments is given in the following paper.

As in the previous experiments, the author is indebted to Dr W G MacCallum, who was kind enough to operate upon the animals required.

EXPERIMENTAL

The conduct of the experiments was similar to those previously reported.² The analytical procedures were the same with the following additions and changes.

Phosphates Titration with uranium acetate, using cochineal as indicator.
Mineral acidity The Folin method.³

Potassium and sodium Fifty cc of urine are ashed, with the aid of a little sulphuric acid, in a platinum dish. The residue is dissolved in water and treated with an excess of barium hydroxide. The precipitate is filtered out and washed thoroughly. A slight excess of sulphuric acid is added to the filtrate. After filtering from the barium sulphate, the liquid is evaporated to dryness in a weighed platinum dish, which is then ignited and weighed (The trace of calcium present is included but may be disregarded). The residue is dissolved in about 20 cc of water, 2 cc of glacial acetic acid are added and the potassium precipitated as the cobaltinitrite by adding 20 cc of the solution of Adie and Wood,⁴ drop by drop (this was found to be necessary to ensure correct results). After standing over night the precipitate is filtered off on a Gooch crucible fitted with a piece of filter paper, and washed with 10 per cent acetic acid, as recommended by Thompson and Morgan,⁵ whose directions are followed from this point. The crucible with the precipitate is placed in the beaker in which the precipitation has been made, an excess of barium hydroxide solution is added and the liquid is boiled for two or three minutes. The precipitate of cobalt hydroxide is filtered off and washed. The filtrate and washings are diluted to a definite volume, generally 250 cc. This liquid is then run into a hot, dilute standard

² Greenwald *loc cit*

³ Folin *Amer Journ of Physiol*, ix, p 265, 1903

⁴ Adie and Wood *Journ of the Chem Soc*, lxxvii, p 1076, 1900

⁵ Thompson and Morgan *Journ of Ind and Eng Chem*, iii, p 398, 1911

solution of potassium permanganate, containing sulphuric acid, until this is decolorized. From the volume required, the amount of potassium is calculated and from this and the weight of the mixed sulphates, the amount of sodium

TABLE I

Excretion of phosphorus after incomplete (?) parathyroidectomy Dog 205

DATE 1912	WEIGHT	VOLUME	SP GR	PHOS PHATES	REMARKS
<i>February</i>	<i>kilos</i>	<i>cc</i>		<i>mgm P</i>	
6	11 67	385		361	
7	11 72	360	1 019	366	
8	11 70	380	1 020	393	
9	11 70	400	1 019	406	
10	11 70	450	1 018	511	
11	11 70	400	1 020	438	
12		415	1 021	467	Parathyroidectomy, 12 noon, Feb 11
13	11 70	400	1 017	353	
14	11 72	330	1 022	324	
15	11 72	380	1 020	445	

At no time did the dog show any evidence of a parathyroid insufficiency. The excretion of phosphorus remained almost unchanged.

TABLE II

Urinary data Dog 209

DATE 1912	VOLUME	SP GR	NITROGEN	CHLORINE	PHOS- PHATES	POTASSIUM	SODIUM
<i>February</i>	<i>cc</i>		<i>grams</i>	<i>mgm</i>	<i>mgm P</i>	<i>mgm</i>	<i>mgm</i>
27	600	1 016	7 567	605	651	1027	370
28	460	1 020	7 741	240	562	727	32
29	400	1 018	6 760	173	493	697	28
<i>March</i>							
1	430	1 017	6 578	163	437	627	54
2	200	1 031	5 675	29	11	618	8
3	420	1 014	5 858	102	14	278	28

The dog weighed 13.50 kilos. Parathyroidectomy was performed at 10.30 a.m., March 1. A slight tremor was observed at 2.30 p.m., March 2. This gradually increased in severity until 10.20 a.m., March 3, when the dog was exsanguinated. In spite of the very marked diminution in the excretion of phosphorus on the day of the operation, the elimination of potassium remained unchanged until the following day.

TABLE III
Urinary data Dog 212

DATE 1912	WEIGHT	VOLUME	SP OR	NITROGEN	PHOS- PHATES	MINERAL ACIDITY
<i>June</i>	<i>kilos</i>	<i>cc</i>		<i>grams</i>	<i>mgm P</i>	<i>cc $\frac{N}{10}$ acid</i>
13	13 55	540	1 026	12 65	746	440
14	13 50	550	1 022	11 38	718	624
15	13 50	490	1 021	9 56	616	595
16	13 50	400	1 024	9 51	639	619
17	13 60	460	1 026	9 40	551	561
18		140*	1 036	4 80	115	127
18 p m		360	1 021	8 34	148	317

* A small quantity of urine was lost

The parathyroids were removed at 11 30 a m, June 17. Symptoms appeared on the afternoon of the following day. The dog was bled to death at 4 p m. On the day of the operation the lessened phosphorus excretion was paralleled by diminished mineral acidity. The latter was increased somewhat on the following day, due, presumably, to a retention of potassium and sodium.

TABLE IV
Urinary data Dog 214

DATE 1912	VOLUME	SP OR	NITROGEN	PHOS- PHATES	CHLORINE	MINERAL ACIDITY
<i>July</i>	<i>cc</i>		<i>grams</i>	<i>mgm P</i>	<i>mgm</i>	<i>cc $\frac{N}{10}$ acid</i>
9	300	1020	6 566	369		
10	360	1020	8 335	511		
11	320	1020	8 170	476		
12			4 960	317		
	430	1026				
13			4 960	317		
14	550	1013	8 880	531	256	435
15	430	1015	7 163	454	241	365
16	350	1020	7 152	425	210	333
17	300	1028	7 290	435	471	96
18	400	1016	8 145	317	162	359
19	350	1021	8 046	348	249	390
20	230	1027	10 000	481	273	453
21	560		8 108	257	245	221
22	450	1016	8 106	147	147	251

The dog weighed 12 40 kilos. Parathyroidectomy was performed at 11 a m, July 16. There was a slight fall in the excretion of phosphorus but no other indication of parathyroid insufficiency. On July 20, at 4 30 p m, both thyroids were removed. The output of phosphorus immediately decreased. Slight twitching was noted at 11 30 p m, July 21. The next

morning, the twitching was more marked and the dog was killed. The very marked drop in the mineral acidity on the day of the first operation cannot, at present, be satisfactorily explained. After the second operation, the mineral acidity fell with the excretion of phosphorus.

TABLE V
Urinary data Dog 215

DATE 1912	NITROGEN	PHOSPHATES	CHLORINE	POTASSIUM	SODIUM
<i>October</i>	<i>grams</i>	<i>mgm P</i>	<i>mgm</i>	<i>mgm</i>	<i>mgm</i>
26	10 73	874	800	823	166
27	8 39	717	533	629	105
28	12 48	1035	924	1282	466
29	8 89	744	382	694	140
30	12 68	1166	1560	1275	519
31	8 38	685	637	619	383
<i>November</i>					
1	10 36	560	1668	869	1143
2	5 08	171	312	166	80
3	14 63	832	1184	791	125
3 p m	7 17	475	629	685	75

The dog weighed 26.0 kilos. Parathyroidectomy was performed in the afternoon of October 31. Slight tremor was observed at 6 p m, November 3. At 8.30 p m, there was severe twitching and, at 9 p m, the dog was killed. On the day of the operation there was a pronounced fall in the elimination of phosphorus. The usual amount of potassium was excreted and the output of sodium was greater than on any other day.

TABLE VI
Urinary data Dog 217

DATE 1912	NITROGEN	PHOSPHATES	POTASSIUM	SODIUM
<i>November</i>	<i>grams</i>	<i>mgm P</i>	<i>mgm</i>	<i>mgm</i>
21	5 479	393	549	291
22	6 340	494	599	268
23	6 803	520	620	277
24	9 202	731	1133	702
25	6 571	533	757	218
26	7 352	242	320	53
27	6 750	310	235	21
27 p m	2 623	113	212	18

The dog weighed 11.0 kilos. The parathyroids were removed on the morning of November 25. At 3 p m November 27, the dog was in tetany and was exsanguinated. In this experiment the excretion of potassium and sodium paralleled that of phosphorus.

For the estimation of phosphorus a slight modification of the Neumann⁴ method was used. It was found that, if Neumann's directions were followed, precipitation of the phosphomolybdate was not always complete. If, however, after oxidation was complete, the acid was diluted and neutralized with ammonium hydroxide, then acidified with nitric acid, heated to about 65°, complete precipitation occurred on addition of ordinary acid molybdate solution and digestion at 65° for one or two hours. The mixture was then allowed to stand at room temperature for several hours, ordinarily over night, then filtered on a Gooch crucible fitted with a piece of hardened filter paper and washed with cold water. The crucible and the precipitate were placed in the beaker in which the precipitation had been made and the precipitate was dissolved in a slight excess of standard sodium hydroxide solution (approximately seventh-normal). From 10 to 30 cc of formalin,⁵ previously neutralized to phenolphthalein with sodium hydroxide, were added, then 10 or 25 cc of standard acid and finally standard sodium hydroxide solution until the liquid was faintly alkaline to phenolphthalein. The volume of the alkaline solution required, less the volume of the acid used, multiplied by the factor, gave the amount of phosphorus in the sample. Even with less than 1 mgm of phosphorus the error is only ± 2 per cent.

For the estimation of the different forms of phosphorus in blood and serum, several methods were tried. Most of these were found to be unsuitable. At first the blood was received in tared flasks containing glass beads and thoroughly shaken. The flasks were weighed and the contents transferred to a bottle containing several volumes of 95 per cent alcohol, recently distilled over sodium hydroxide. After standing a few days, the mixture was filtered through a linen bag and the residue extracted with hot alcohol in a continuous extraction apparatus. It was found that the extracts were always deeply colored and that they contained considerable quantities of material (hematin?) which made subsequent separation of the lipoids, either by precipitation with chloroform⁶ or by extraction with anhydrous ether, difficult and inaccurate.

⁴ Neumann *Zeitschr f physiol Chem*, xxxvii, p 115, 1903

⁵ Bang *Biochem Zeitschr*, xxxii, p 443, 1911

⁶ Koch and Woods *this Journal*, i, p 206, 1906

An attempt was made to dry the blood by mixing it with infusorial earth to form a thick paste and exposing this to the air, or *in vacuo*, at a moderate temperature (30–35°). A fine, dry powder was readily obtained but it was found that extraction with the usual solvent, removed only a small portion of the phospholipins known to be present.

For precipitation of the proteins and extraction of the lipoids, acetone was found to be most suitable. Addition of four volumes of acetone to blood or serum precipitates protein and inorganic phosphates completely. Subsequent treatment of the precipitate with hot acetone removes the lipoids practically completely but does not affect hemoglobin nor dissolve inorganic phosphate. If the acetone extract be evaporated and the lipoids precipitated with chloroform, only traces of phosphorus are found in the filtrate. After extraction with acetone, treatment with hot alcohol and ether removes, at most, only traces of phosphorus compounds from serum and only comparatively small amounts from whole blood.

For the determination of water-soluble phosphorus, direct extraction of the residue from the lipid extraction with hot water and dialysis were tried. Both were failures, removal of inorganic phosphate being incomplete within any reasonable period. Extraction with dilute hydrochloric acid was also unsuccessful, for the protein swelled to a jelly. Direct estimation of the non-colloidal phosphorus of serum was attempted in the filtrate from the kaolin precipitate produced by the method of Michaelis and Rona,⁷ but it was found that all of the inorganic phosphate added could not be recovered. Apparently some had been adsorbed.

The procedures that give the best results in the estimation of water-soluble phosphorus are the following:

- 1 The serum or blood is mixed with nine or nineteen volumes, respectively, of a solution containing 1 per cent of acetic acid and 0.5 per cent of picric acid. After a few hours the liquid is filtered and the phosphorus in an aliquot portion of the filtrate is estimated in the usual manner.

- 2 The dry residue from the lipid extraction is ground to a powder and treated with a dilute solution of hydrochloric acid (1

⁷ Michaelis and Rona. *Biochem. Zeitschr.*, vii, p. 329, viii, p. 356, 1908.

per cent by volume of the concentrated acid) saturated with picric acid Ten or twenty volumes, respectively, of this solution are used for each volume of serum or blood under examination The mixture is shaken at intervals and allowed to stand over night It is then filtered and phosphorus is estimated in an aliquot portion of the filtrate

Concordant values for the phosphorus precipitated from these extracts by barium and ammonium hydroxides were not obtained, consequently only total phosphorus was estimated

That these methods are fairly accurate is shown by the figures given in tables I-III

As already stated, separation of the phosphorus compounds of the blood into various fractions was not successful in the first series of experiments The figures given in table IV were obtained by adding together the values obtained for the phosphorus in the different fractions In the case of dog 111, however, the total phosphorus was estimated directly As can readily be seen, the phosphorus content of the blood of the parathyroidectomized dogs was greater than in that of the control animals

In the two following experiments, the blood was mixed with infusorial earth and dried On subsequent extraction, phospholipins were not removed by the usual solvents However, determinations of the total solids, nitrogen and phosphorus were made with the results indicated in table V Six months later, samples of this dry powder were extracted with the picric-hydrochloric acid mixture and phosphorus estimated in the extract The values obtained are given in the table

The material for the next series of analyses consisted of defibrinated blood from a normal and a parathyroidectomized dog, obtained in the course of other experiments Data relating to the sex, weight and food of the dogs are lacking In this series the acetone extraction method was used but the residues were wasted in a vain attempt to remove inorganic phosphate by dialysis or by means of boiling water However, direct estimations of the acid-extractable phosphorus were made, using the acetic acid-picric acid mixture already described The figures obtained are given in table VI

The blood from the next pair of dogs was allowed to clot The serum was centrifuged to remove all the cells and then analyzed Unfortunately, scarcity of material and a series of accidents made

it impossible to determine more than the total nitrogen and phosphorus and the phosphorus extracted by acetone and by subsequent treatment with absolute alcohol and ether. The figures obtained are presented in table VII.

Because parathyroidectomy was not followed by symptoms of parathyroid insufficiency, the next dog, 214, had both thyroids removed. Tetany then developed and the dog was bled. The blood, as also that of the control, was oxalated. In calculating the results of the analyses, which are summarized in table VIII, proper allowance has been made for the weight of the ammonium oxalate solution used.

In the three following experiments, perfectly clear serum, almost free from hemoglobin, was obtained. If the serum is at all red, it contains enough inorganic phosphate derived from erythrocytes to make analysis useless. The results obtained are summarized in table IX.

As can readily be seen on examining the tables, the total amount of phosphorus in the blood and serum of parathyroidectomized dogs is regularly greater than in normal dogs. This increase is not associated with a corresponding increase in the amount of total solids or of nitrogen, on the contrary it may be very marked in the presence of an equally pronounced diminution of total solids and nitrogen. The increase seems to be due largely, if not entirely, to an increase in that form of phosphorus which, for want of a better name, we have called acid-extracted phosphorus. A part of this fraction is inorganic phosphate, whether or not all of it is inorganic, cannot be stated. The amount of lipin-phosphorus in the blood and serum of dogs varies considerably and, apparently, bears no relation to the presence or absence of the parathyroid glands.

Very recently Juschtschenko⁸ has published the results of an investigation into the nitrogen and phosphorus content of the tissues of normal and parathyroidectomized dogs. Only his results with serum need concern us now. He dried the serum, first at 30°, then at 65°, and estimated inorganic phosphate by the method of Stutzer and Neumann. He states that the amount of inorganic phosphate of the serum is the same in dogs in tetany and in normal dogs. He makes no reference whatever to his

⁸ Juschtschenko *Biochem Zeitschr*, xl, p 64, 1912

earlier paper³ in which he claimed that the blood of dogs and rabbits in tetany after thyroidectomy contained more inorganic phosphate than did the blood of normal animals. It is also interesting to note that, in the two instances in which he determined the phosphorus in the residue obtained after extraction of the lipoids from the dried serum, the higher values were obtained in the preparations from parathyroidectomized dogs. The conclusions drawn by Juschtschenko from his experiments are open to other objections. His dogs were in tetany for hours and days before they were bled so that it is impossible to distinguish the effect of the parathyroidectomy from the possible effect of the tetany and inanition. Phospholipins are apt to decompose when kept at 65° for twenty-four hours, as occurred in Juschtschenko's method of drying the serum. Apparently he did not attempt to test the accuracy of his method, to see if inorganic phosphate added to the serum could be recovered.

SUMMARY

After removal of the parathyroid glands, the total phosphorus of the blood and serum of dogs is increased. This may be observed at a time when the tremor is still very slight. The increase may be as much as 160 mgm of phosphorus per kilo of blood. The greater part of this increase is in the fraction which is insoluble in the usual lipid solvents but is soluble in a mixture of dilute hydrochloric or acetic and picric acids.

It is with great pleasure that I acknowledge my indebtedness to Dr W G MacCallum, who was kind enough to remove the parathyroids from the animals required.

TABLE I

Acid-extractable phosphorus in 25 cc of ox-blood

(Results expressed as cubic centimeters of standard solution required for titration of the phosphomolybdate)

EXTRACTED WITH DILUTE ACETIC AND PICRIC ACIDS	EXTRACTED WITH HYDROCHLORIC AND PICRIC ACIDS AFTER EXTRACTION WITH ACETONE
cc	cc
20 45	20 40
20 50	20 50
	20 50

³ Juschtschenko *Zeitschr f physiol Chem*, lxxv, p 141, 1911

TABLE II

Recovery, by the picric-acetic acid method, of inorganic phosphate added to blood

($Mg_3P_2O_7$ dissolved in dilute HNO_3 , neutralized with NaOH before adding to the blood Results expressed as cubic centimeters of standard solution required for titration of the phosphomolybdate)

NUMBER	BLOOD	ADDED PHOSPHATE	BLOOD AND PHOSPHATE	PHOSPHATE RECOVERED
	cc	cc	cc	cc
1	15 17	49 85	64 00	48 83
	15 17			
2	20 45	49 85	70 50	50 02
	20 50	49 85	70 70	50 22
		49 85	70 10	49 62
3	26 4	66 0	92 2	65 8
	26 2	66 0	92 0	65 8

TABLE III

Recovery, by the picric-hydrochloric acid method, of inorganic phosphate added to blood

($Mg_3P_2O_7$ dissolved in dilute HNO_3 , neutralized with NaOH before adding to blood Results expressed as cubic centimeters of standard solution required for titration of the phosphomolybdate)

ACETONE-SOLUBLE PHOSPHORUS		ADDED PHOSPHATE	ACID EXTRACTED PHOSPHORUS		PHOSPHATE RECOVERED
Blood	Blood and phosphate		Blood	Blood and phosphate	
cc	cc	cc	cc	cc	cc
11 45	11 60	49 95	20 40	69 60	49 20
	11 30	49 85	20 50	70 45	49 95
		49 85	20 50	71 33	50 83

TABLE IV

Total phosphorus in blood from normal and parathyroidectomized dogs

NUMBER	PARATHYROIDECTOMIZED	NUMBER	CONTROL
	mgm per kilo		mgm per kilo
105	477		
107	521	108	425*
110	512	109	416
111	474†		

Parathyroids removed ten days before bleeding no symptoms
† Chronic latent tetany See protocol of experiment below

Dog 105 Fox-terrier, male, weight 10 70 kilos The parathyroids were removed at 10 a m , January 19, 1911 On the following day, slight twitching was noticed at 2 p m This was more marked at 3 p m , when the dog was exsanguinated

Dog 107 Mongrel, bitch, weight 9 20 kilos Parathyroidectomy was performed at 2 p m , January 31, 1911 Slight twitching was noticed at 2 30 p m , February 1, and at 3 30 the dog was bled

Dog 108 Mongrel, male, weight 8 35 kilo Four parathyroids were removed on February 4, 1911 No symptoms appeared and the dog was bled at 3 p m , February 14

Dog 109 Bull-terrier, bitch, weight 8 30 kilos Exsanguinated at 4 30 p m , February 11, 1911

Dog 110 Beagle, bitch, weight 9 60 kilos Parathyroidectomy was performed at 3 p m , February 10, 1911 Very slight twitching was noticed at 3 30 p m , February 12 This gradually became more marked until, at 5 30 p m , the dog was exsanguinated

Dog 111 Bull-terrier, bitch, weight 13 0 kilos Parathyroidectomy was performed on March 21, 1911, but no symptoms of parathyroid insufficiency appeared within the following two weeks On April 6, a pup was born prematurely and died the same day Another was born the following morning and lived about twenty-four hours On the morning of April 8, a slight tremor was noticed This grew more marked and continued several days Two pups were found in the cage on the morning of April 10 They were quite well developed but the mother paid little attention to them and they died the following morning On this day (April 11), four pups made their appearance The mother suckled them but one died on the 16th and another on the 17th The other two survived and were raised to an age of about four months when they were unfortunately lost They seemed to be quite normal The mother continued in tremor until April 21, two weeks in all In June, attempts were made to induce tetany by feeding large quantities of meat and of extract of beef These were not successful After a return to the usual diet for three days, the dog was bled at 3 p m

TABLE V

Analyses of blood from a parathyroidectomized dog (209) and from a normal dog (210)

	209	210
Total solids	17 80 per cent	24 26 per cent
Total nitrogen	2 31 per cent	3 16 per cent
Total phosphorus	592 7 mgm per kilo	430 3 mgm per kilo
Phosphorus extracted with dilute acid	387 4 mgm per kilo	290 4 mgm per kilo

Dog 209 Bull-terrier, male, weight 13 50 kilos The parathyroids were removed at 10 30 a m , March 1, 1912 A slight tremor was noticed at 2 30 p m , the following day This became a little more marked during the afternoon but was not severe at 2 30 a m , March 3 There was pronounced twitching at 10 20 a m and the dog was bled to death

Dog 210 Bull-terrier, male, weight 13 00 kilos Exsanguinated at 9 a m , March 8

TABLE VI

Analyses of blood from a parathyroidectomized dog and a normal dog

	PARATHYROIDECTOMIZED	NORMAL
Total nitrogen	3 02 per cent	2 97 per cent
Total phosphorus	482 mgm per kilo	409 mgm per kilo
Phosphorus extracted with acetone	145 mgm per kilo	135 mgm per kilo
Phosphorus extracted with alcohol and ether	11 7 mgm per kilo	12 2 mgm per kilo
Phosphorus extracted with picric-acetic acid solution	284 mgm per kilo	239 mgm per kilo

TABLE VII

Analyses of serum

	PARATHYROIDECTOMIZED DOG 212	NORMAL DOG 213
Total nitrogen	9 833 gm per kilo	9 350 gm per kilo
Total phosphorus	209 mgm per kilo	179 mgm per kilo
Phosphorus extracted with acetone	129 mgm per kilo	133 mgm per kilo
Phosphorus extracted with alcohol and ether	Trace	Trace

Dog 212 Bull-terrier, bitch, weight 13 50 kilos The parathyroids were removed at 11 30 a m , June 17, 1912 At 4 p m , the following day, the dog was in tetany and was exsanguinated

Dog 213 Mongrel, bitch, weight 11 60 kilos Bled at 4 p m , June 22, 1912

TABLE VIII

Phosphorus in blood from a thyro-parathyroidectomized dog (214) and a normal dog (214B)

	MILLIGRAMS PHOSPHORUS PER KILO OF BLOOD	
	214	214B
Total	436	370
Extracted with acetone	162	140
Extracted with alcohol and ether	8	7
Extracted with picric-hydrochloric acid	233	192
Not accounted for (protein phosphorus?)	33	31

Dog 214 Mongrel, male, weight 12.40 kilo. Parathyroidectomy was performed July 16, 1912. Tremor did not appear, nor was the excretion of phosphorus diminished. At 4.30 p.m., July 20, both thyroids were removed. At 11.30 p.m., the following day, there was slight, occasional twitching. This was more evident at 9 a.m. the next morning and the dog was bled to death.

Dog 214B Mongrel, male, weight 13.50 kilos. Bled at 9 a.m., August 9, 1912.

TABLE IX

Phosphorus in serum from parathyroidectomized and normal dogs

NUMBER	PHOSPHORUS PER KILO OF SERUM			
	Total	Acetone extract	Acid extract	Residue
	mgm	mgm	mgm	mgm
Parathyroidectomized				
215	212	149	62.3	
217	222	128	76.7	
219*	291	194	87.7	3.8
Normal				
216	157	131	26.9	
218	244	186	44.4	2.4
220	167	110	45.5	

*Complete thyroidectomy

Dog 215 St. Bernard, male, weight 26.0 kilos. The parathyroids were removed in the afternoon of October 31, 1912. Slight tremor appeared at 6 p.m., November 3. At 8.30, twitching was quite pronounced and the dog was bled.

Dog 216 Mongrel, male, weight 14 0 kilos Bled at 8 p m , November 16, 1912

Dog 217 Bull-terrier, male, weight 11 0 kilos Parathyroidectomy was performed on the morning of November 25, 1912 Tremor was doubtful at 9 a m , November 27 but, at 3 p m , the dog had a severe attack of tetany and at 3 45 was exsanguinated

Dog 218 Mongrel male, weight 9 45 kilos Bled at 4 30 p m , February 12, 1913

Dog 219 Mongrel, male, weight 14 7 kilos Four parathyroids were removed on December 19, 1912 As no symptoms appeared, the right thyroid was removed on December 22 This was also without apparent effect and at 3 p m , December 25, the other thyroid was removed At 8 p m , December 29, there was slight, though unmistakable, twitching No change was observed at 10 30 p m and the dog was bled A severe attack of tetany occurred during the bleeding

Dog 220 Mongrel, male, weight 16 0 kilos Bled at 10 30 p m , January 12, 1913

RESEARCHES ON PURINES

ON 2-METHYLMERCAPTO-6,8-DIOXYPURINE AND 2-METHYLMERCAPTO-6-OXY-8-AMINOPURINE

(TENTH PAPER)¹

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(Received for publication, March 21, 1913)

The only method hitherto employed for the preparation of alkylmercapto purines has been to alkylate thiopurines by means of alkyl halides. Thus, Fischer² obtained 6-methylmercapto-7-methylpurine (I) by the action of methyl iodide on the potassium salt of 6-thio-7-methylpurine.

As far as we are aware no alkylmercapto-orthodiaminopyrimidines have hitherto been prepared.³ We have succeeded in preparing 2-methylmercapto-4,5-diamino-6-oxypyrimidine (V) and have used it for the synthesis of purine derivatives. The reactions leading to the preparation of this diaminopyrimidine are as follows. 2-Thio-4-amino-6-oxypyrimidine (II)⁴ was alkylated in a solution of potassium hydroxide at room temperature by means of dimethyl sulphate. This reaction had previously been carried out with methyl iodide but the reaction was not very smooth.⁵ The yield of 2-methylmercapto-4-amino-6-oxypyrimidine (III) obtained by the use of dimethyl sulphate was as high as 90 per cent of the calculated. This mercapto derivative then gave a quantitative yield of 2-methylmercapto-4-amino-5-nitroso-6-oxypyrimidine (VI) when acted on by nitrous acid. The nitroso compound was reduced smoothly to 2-methylmercapto-4,5-diamino-6-oxypyrimidine (V),

¹ Johns, this *Journal*, **xiv**, p 1, 1913

² Emil Fischer, *Ber d deutsch chem Gesellsch*, **xxxi**, p 437, 1898

³ Some preliminary experiments on this work were made in 1906. Johnson, Johns and Heyl, *Amer Chem Journ*, **xxxvi**, p 172, 1906

⁴ W. Traube, *Ann d Chem (Liebig)*, **cccxxi**, p 71, 1904

⁵ Johnson and Johns, *Amer Chem Journ*, **xxxiv**, p 181, 1905

using ammonium sulphide as the reducing agent The yield was 90 per cent of theory

When 2-methylmercapto-4,5-diamino-6-oxypyrimidine was heated with urea it gave 2-methylmercapto-6,8-dioxypurine (IV), the yield being 60-70 per cent of the calculated The mercapto group in this purine is very firmly bound but on boiling with a large excess of 20 per cent hydrochloric acid for twenty hours, methylmercaptan was evolved slowly and uric acid (VII) was formed

It is well known that when orthodiaminopyrimidines are heated with thiourea 8-thiopurines are formed Thus, when 2-thio-4,5-diamino-6-oxypyrimidine (IX)⁶ was heated with thiourea 2,8-dithio-6-oxypurine (XII)⁷ resulted The presence of the methylmercapto group in position 2 instead of the sulphur renders 2-methylmercapto-4,5-diamino-6-oxypyrimidine less active towards thiourea Instead of obtaining the expected 2-methylmercapto-6-oxy-8-thiopurine we obtained 2-methylmercapto-6-oxy-8-aminopurine (VIII) It is possible that traces of the 8-thiopurine were also formed although we did not detect any of this compound The yield of the 8-aminopurine was 60 per cent of the calculated This result may be explained by assuming that the thiourea formed guanidine thiocyanate which then reacted with the diaminopyrimidine This view was confirmed by heating the diaminopyrimidine with guanidine thiocyanate which also gave 2-methylmercapto-6-oxy-8-aminopurine The same result was also obtained by using ammonium thiocyanate instead of guanidine thiocyanate

These results are interesting in view of the fact that the writer has attempted to obtain 8-aminopurines by heating other diaminopyrimidines with salts of guanidine but without success Even when 2-thio-4,5-diamino-6-oxypyrimidine is heated with guanidine thiocyanate it fails to give an 8-aminopurine

When 2-methylmercapto-6-oxy-8-aminopurine was heated with hydrochloric acid the amino group was removed before the methylmercapto group On prolonged heating uric acid was obtained

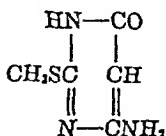
2-Alkylmercapto-6-oxypyrimidines (X)⁸ react readily with phosphorus halides to form 6-chlor derivatives (XI) We intend to

⁶ W Traube *Ann d Chem* (Liebig), cccxxxi, p 75, 1904

⁷ Johns and Hogan *this Journal*, xiv, p 299, 1913

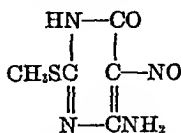
⁸ Wheeler and Johnson *Amer Chem Journ*, xlix, p 496, 1903

EXPERIMENTAL PART

*2-Methylmercapto-4-amino-6-oxypyrimidine*⁹

This compound was previously prepared by the action of methyl iodide on the sodium salt of 2-thio-4-amino-6-oxypyrimidine. We find that dimethyl sulphate can be used instead of methyl iodide. The yield is greater, the reaction requires less time and the cost is less. The methylation was performed as follows:

Twenty-five grams of pulverized 2-thio-4-amino-6-oxypyrimidine were dissolved in 100 cc. of a 10 per cent solution of potassium hydroxide, care being taken to avoid an excess of the alkali. Twenty-five grams of technical dimethyl sulphate were then added in small portions, with thorough shaking after each addition. In some cases it was found necessary to dilute with water as the precipitate which resulted became too thick to permit thorough mixing to take place. After the mixture had stood at room temperature for fifteen minutes it gave an acid reaction and the precipitate was filtered by suction. The mercapto pyrimidine thus obtained was removed to a flask while still moist, 200 cc. of 95 per cent alcohol were added and the mixture was heated to the boiling point of the alcohol. This dissolved most of the precipitate. The flask was then cooled and allowed to stand at room temperature for an hour. On filtering, 20 to 25 grams of pure 2-methylmercapto-4-amino-6-oxypyrimidine were obtained. This is 75 to 90 per cent of the calculated weight. When this was mixed with a pure sample of 2-methylmercapto-4-amino-6-oxypyrimidine obtained by alkylating with methyl iodide the melting point of the mixture was 267°C. This is the melting point of the pure substance.

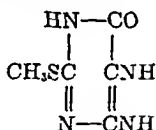
2-Methylmercapto-4-amino-5-nitroso-6-oxypyrimidine

⁹ Johnson and Johns *Amer. Chem. Journ.*, xxxiv, p. 181, 1905

Twenty grams of 2-methylmercapto-4-amino-6-oxypyrimidine were dissolved in 350 cc of water containing 5.1 grams of sodium hydroxide. A solution of 10 grams of sodium nitrite in 50 cc of water was added. The mixture was then acidified by the gradual addition of 17 grams of glacial acetic acid. The precipitate which formed was white but turned blue in a short time. The mixture was allowed to remain at room temperature over night after which the precipitate was filtered off, washed with cold water and used, without drying, for the preparation of 2-methylmercapto-4,5-diamino-6-oxypyrimidine. The yield of the nitroso derivative was almost quantitative. It was but slightly soluble in hot water or alcohol and was not soluble in benzene. It formed a red solution in alkalis and blue in acids. A portion was purified for analysis by dissolving it in ammonia and precipitating with acetic acid. The substance did not melt but began to decompose at about 255°C.

N	Calculated for	Found	
	$C_5H_7O_2N_3S$	I	II
	30.10	29.75	30.16

2-Methylmercapto-4,5-diamino-6-oxypyrimidine



Fifty cubic centimeters of a 10 per cent solution of ammonium sulphide were placed in a 1-liter flask and heated on the steam bath. The moist 2-methylmercapto-4-amino-5-nitroso-6-oxypyrimidine obtained in the previous experiment was added gradually. Ammonium sulphide was also added when the solution turned red as this indicated that the nitroso compound was present in excess. When the ammonium sulphide was present in excess the solution was yellow. When all of the nitroso compound has been reduced the addition of excess of ammonium sulphide should be avoided or the diamino compound obtained will be highly colored. Sulphur separated after the reduction had proceeded for a few minutes. When the reduction was complete the mixture was concentrated by boiling in a casserole over a free flame until the separation of sulphur ceased. The solution was then filtered

rapidly to remove the sulphur. The filtrate on cooling gave an almost colorless, crystalline precipitate. A second crop was obtained by concentrating the mother liquor on the steam bath. The diaminopyrimidine was dried at 30–40°C. Drying at high temperatures causes it to become black. When the reduction was carried out carefully the diamino derivative was almost pure. It began to shrink at about 198°C and melted with decomposition at 211°C. It was easily soluble in hot and appreciably soluble in cold water and moderately soluble in hot alcohol but was not soluble in boiling benzene. The yield based on the 2-methylmercapto-4-amino-6-oxypyrimidine used was over 90 per cent of the calculated.

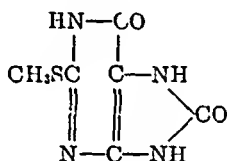
N

Calculated for
C₄H₆ON₂S

32.53

Found
32.13

2-Methylmercapto-6,8-dioxypurine



Five grams of urea and an equal weight of 2-methylmercapto-4,5-diamino-6-oxypyrimidine were intimately mixed by pulverizing together in a mortar. This mixture was heated in an oil bath at 165°C for fifteen minutes and then at 170–180°C until a hard cake had formed, about one hour in all. The reaction product was crushed and dissolved in hot dilute sodium hydroxide and the solution was clarified with blood coal. The purine was precipitated from the hot solution by acidifying with dilute hydrochloric acid. The yield was 3.5 grams or 60 per cent of the calculated weight. The purine was obtained in a finely divided, granular form. One part by weight required a little more than 100 parts of boiling water to effect solution. It was almost insoluble in alcohol, benzene, or glacial acetic acid. It did not dissolve readily in ammonia but was easily soluble in dilute sodium hydroxide. It dissolved with effervescence in concentrated nitric acid and gave the murexide reaction. It did not decompose at 320°C.

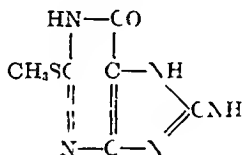
	Calculated for $C_4H_4O_2N_2S$	Found
N	28 28	28 17

Conversion of 2-methylmercapto-6,8-dioxypurine to uric acid

One gram of 2-methylmercapto-6,8-dioxypurine was boiled with 150 cc of 20 per cent hydrochloric acid under a return condenser for twenty hours. At first complete solution of the mercapto purine took place but after boiling for several hours uric acid began to precipitate. The reaction mixture was evaporated to dryness and the residue was dissolved in dilute sodium hydroxide, the solution filtered and acidified with hydrochloric acid whereupon uric acid precipitated.

	Calculated for $C_5H_4O_2N_4$	I Found	II
N	33 33	33 37	33 36

2-Methylmercapto-6-oxo-8-aminopurine



This purine was obtained by heating one part by weight of 2-methylmercapto-4,5-diamino-6-oxypyrimidine with an equal weight of either thiourea or ammonium thiocyanate but was best prepared as follows.

Five grams of the mercaptodiaminopyrimidine and 5 grams of guanidine thiocyanate were pulverized together and the mixture was heated at 180°C in an oil bath for two hours. During the heating the mixture melted partly, foaming ensued and, finally, a hard crust was formed. The reaction product was treated with cold water to remove an excess of guanidine thiocyanate, the residue was dissolved in dilute sodium hydroxide and the solution was clarified with blood coal. On acidifying the hot solution with acetic acid the purine was obtained as a fine powder. The yield was 60 per cent of the calculated. The substance did not decompose at 320°C. It dissolved in about 300 parts of boiling water. It was slightly soluble in hot alcohol or glacial acetic acid and insol-

uble in benzene It did not dissolve very readily in ammonia It gave a brilliant murexide reaction When boiled with hydrochloric acid the amino group was hydrolyzed off before the mercapto group Boiling with 10 per cent sodium hydroxide did not remove either the amino group or mercapto group

	Calculated for $C_8H_7ON_4S$	Found	
		I	II
N	35 53	35 44	35 67

When the above mercaptoaminopurine was boiled for twenty hours with 20 per cent hydrochloric acid, uric acid was obtained Analyses on different samples resulted as follows

	Calculated for $C_5H_4O_3N_4$	Found	
		I	II
N	33 33	33 14	33 58

ON THE EXCRETION OF NITROGEN SUBSEQUENT TO LIGATION OF SUCCESSIVE BRANCHES OF THE RENAL ARTERIES

By J D PILCHER

(From the Pharmacological Laboratory, Western Reserve University Medical School)

(Received for publication, March 22, 1913)

MacNider¹ has shown that ligation of the posterior branch of the renal artery leads to necrosis of practically the posterior third of the kidney. This is followed by partial regeneration of the renal epithelium and glomeruli, reaching its height about the twenty-sixth to the thirtieth day, subsequently atrophy of the new tissue follows as a result of fibrosis of the stroma. Further, a collateral circulation develops in the area supplied by the ligated branch, first in the medulla, then at the cortex-medullary boundary zone and finally the cortex, usually in the form of vascular streaks. This collateral circulation takes place between the arteriolae rectae and pseudo-arteriolae rectae of the ligated and unligated vessels. Normally the pelvis and a portion of the renal papillae receive a partial blood supply through vessels entering the kidney with the ureter, there is a further anastomosis between the vessels of the capsule of the kidney and the recurrent suprarenal and phrenic arteries.

The following description is a report of two observations made as an attempt to determine whether there are any changes in the total nitrogen excretion incident to the renal changes. The effect of excision of various segments of the kidney has been studied by several observers, notably, Bradford,² Bainbridge and Beddard³ and Pearce.⁴ All agree that removal of three-fourths of the total

¹ W de B MacNider *Journ of Med Res*, xxiv, p 425, 1911

² J R Bradford *Journ of Physiol*, xxiii, p 415, 1898-9

³ F A Bainbridge and A P Beddard *Proc Roy Soc* lxxix, p 75, 1907

⁴ R M Pearce *Journ of Exp Med*, x, p 632, 1903

kidney substance results in death within a few weeks. In such animals Bradford found an increased output of nitrogen (as urea). This observation was not confirmed by other investigators, who were, therefore, unable to agree with Bradford's suggestion that the kidney has an influence on nitrogenous metabolism, but attributed the increased nitrogen excretion to inanition only. The present method, by minimizing complicating operative factors, offered a better chance of solving the effect of throwing a part of the kidney out of function.

Methods The observations were made on one cat and one dog, both full-grown. The cat was fed hashed beef and the dog, ordinary dog biscuit, the nitrogen content of which was determined by the Kjeldahl method. The animals were kept in the usual metabolism cages. The urine was examined daily with the cat, and two or three times weekly with the dog, but daily, shortly before operations. The urines were preserved in strong sulphuric acid when kept longer than twenty-four hours. After determining the nitrogen excretion for suitable periods (two weeks with the cat, three to five with the dog) successive branches of the renal arteries were ligated under anesthesia⁵ as follows:

Ligations on the cat

December 29, 1909 First operation: ligated branch of left renal artery.

January 12, 1910 Second operation, fourteen days later: ligated and cut the upper branch of the right renal artery.

January 26, 1910 Third operation, fourteen days later: ligated and cut the left renal artery, as the remaining branch could not be located. Following this operation the cat temporarily lost greatly in weight and ate but a fraction of its usual food, without evident cause (there seemed to be no sepsis). Observations were discontinued until the following winter.

February 9, 1911 Fourth operation, twelve months after ligating the left renal artery and thirteen months after the first operation, during which period the cat increased in weight from 3300 grams to 3800 grams: ligated and cut the lower branch of the right renal artery. The cat died in twenty-four hours, after severe nausea, vomiting, great prostration and subnormal temperature. At autopsy no peritonitis was present, the left kidney was atrophied (the artery was ligated one year previously), the right kidney

⁵ The dog was etherized, the cat received 3 cc per kilogram of the following solution *per rectum* (supplemented by ether): atropine sulphate, 0.02 gram, morphine sulphate, 1.0 gram, urethane, 20.0 grams, aqua, 100 cc.

was adherent to the abdominal wall, but the capsule was very easily stripped from the cortex, it was practically normal in size, with the upper pole somewhat smaller than the lower (the upper renal branch had been ligated thirteen months previously)

Ligations on the dog

April 17, 1910 First operation ligated lower branch of the left renal artery Following the operation there was a local infection and considerable prostration Recovery was complete in five or six weeks

June 24, 1910 Second operation, seventy days later ligated one branch of right renal artery, this operation was also followed by considerable prostration, without infection, and observations were discontinued

December 5, 1910 Third operation, seven months after the first and five months after the second ligated upper branch of left kidney Following the operation there was severe prostration similar to the cat, as described above, death in forty-eight hours At autopsy there was no peritonitis, the right kidney was atrophied but there was one small patent arterial branch running to it, the left kidney was normal in size but the lower pole was somewhat smaller than the upper

Table of ligations and N intake and output (in urine)

LIGATION OF BRANCHES OF RENAL ARTERIES	TOTAL KIDNEY SUBSTANCE WITH NORMAL BLOOD SUPPLY	TOTAL N		
		Intake <i>grams</i>	Output <i>grams</i>	Retention <i>per cent</i>
<i>Dog</i>				
	4/4	4 46	3 77	15 4
Left lower branch	3/4	4 46	3 75	15 0
Entire right artery	1/4	4 46	3 25	27 1
<i>Cat</i>				
	4/4	3 15	3 25	-3 1
Left branch	3/4	3 18	3 22	-1 2
Right branch	1/2	2 96	2 86	3 3
Entire left artery	1/4	2 88	2 79	3 1

The nitrogen excretion in the cat The average daily excretion was determined during the different periods of observation, each of fourteen days' duration As is shown by the accompanying table the excretion remained practically unchanged after the first ligation, the daily output exceeding the intake by 0.04 gram Subsequent to the second ligation, that is, with one branch of each

renal artery ligated, the second excretion was but 0.10 gram (3.5 per cent) less than the intake. One entire renal artery was then ligated, leaving the kidney tissue supplied by but one (of a total of four) arterial branch. As was stated above, this resulted in severe prostration and anorexia, the daily nitrogen output (2.33 grams) exceeding the intake (1.58 grams) by 0.75 gram, or about 30 per cent. The severe prostration and markedly increased nitrogen excretion corresponds quite closely to the observations of others, mentioned above, on removal of three-quarters of the total kidney substance. However, in this case the animal made a perfect recovery after a few weeks, and it may be that ligation of one entire renal artery and one branch on the opposite side did not destroy the function of quite three-quarters of the total renal substance, or there may have been a development of a collateral circulation from the capsule or between the smaller vessels of the ligated and unligated branches, which was sufficient to preserve complete loss of function of the area nourished by the ligated branch. With the establishment of the collateral circulation there may have been regeneration of renal tissue in case of atrophy immediately subsequent to the ligation. During a period of twelve days, one year later, the cat was practically in nitrogen equilibrium, the daily intake exceeding the output by but 0.09 gram. Inasmuch as death ensued so promptly (in about twenty-four hours) on ligation of the last remaining arterial branch it follows that, in this case, occlusion of one branch of the renal artery does not result in sufficient collateral circulation from the capsule to preserve the function of the ligated area, so that, after one year, it is unable to preserve life, even for a short time, if the renal artery is then ligated.

The nitrogen excretion in the dog The nitrogen intake remained constant throughout the periods of observation (4.46 grams daily). There was a deep-seated infection following the first and considerable *prostration* after the second ligation⁶ so that observations were not made until the dog returned to its normal condition. One month after the first ligation the dog was in normal condition and during a period of thirty-five days, excreted the same daily quantity of nitrogen (3.75 grams) as before operation. Dur-

⁶ At this period probably one entire artery and one branch of the opposite artery had been ligated.

ing this period there was a 35 per cent gain in weight as against a pre-operative gain of 17 per cent. The next observations were made during a period of thirty-seven days, four months after the second operation, at a time when one kidney had practically atrophied and one branch of the opposite side had been ligated for about seven months, the average daily nitrogen excretion fell 13 per cent below the normal excretion. During this period (37 days) the dog lost about 35 per cent in weight. With considerably less than one-half of the normal kidney substance functioning the dog was apparently in perfect condition and able to excrete approximately (13 per cent less) the normal quantity of nitrogen, perhaps there was some deficiency in nitrogen excretion since there was a retention with a loss in weight.

Inasmuch as the dog died within forty-eight hours after ligation of the last renal arterial branch, and without sepsis, it can be deduced that, during the seven months' interval between the ligation of the two branches, as in the cat, there was not formation of sufficient collateral circulation from the capsule to preserve the function of the ligated areas.

The profound prostration following the second ligation was undoubtedly due to removing the function of the entire kidney for this kidney was atrophied at autopsy, so that practically but one-fourth of the entire kidney substance was functioning at this time (as one branch of the opposite side had previously been ligated). This prostration was probably accompanied by a marked temporary nitrogen excretion, as was noted in the cat under similar conditions and observed by others when three-fourths of the kidney substance was removed at one time.

The daily nitrogen excretion in the cat immediately following the ligation. Subsequent to the first two ligations the output was either normal (after the first) or somewhat below normal for a day or two, then for a period of four or five days the output was increased to 13 per cent (first operation) and 11 per cent (second operation) above the intake, in each case during the eight days until the next operation the output fell slightly below the intake. Following the third ligation (one entire artery) the excretion was increasingly greater than the intake during the observation period of fourteen days, when the animal lost weight and the nitrogen intake was below normal.

Studying the influence of ether anesthesia upon the excretion of nitrogen in dogs, Hawk⁷ reports an increased nitrogen output following the anesthesia which may persist for several days. As the results in the cat agree with Hawk's observations on dogs, the temporary increased nitrogen excretion following the ligation may be attributed to the anesthetic in part at least.

The quantity of urine With the greater part of the kidney substance removed, Bradford's dogs excreted permanently a volume of urine much above the normal, and of low specific gravity. Bainbridge and Beddard were unable to confirm his results but report the usual quantity and specific gravity of urine. The results in this series agree with those of the latter observers. The dog excreted practically the same volume of urine throughout, from 100–150 cc daily, no observations on the total volume of the cat's urine were made until after the second operation when the quantity remained constant (75 cc) until the final operation (75 cc is not an abnormally large quantity of urine for a cat of 3300 grams). At times immediately following operation the water intake and output were increased in quantity.

Just before the final operation the urine of both animals was free from albumen and casts of any kind.

Partial nephrectomy in dogs has been reported to be followed by considerable rise in blood pressure,⁸ it would seem that such increased pressure probably would be associated with cardiac hypertrophy. In this work no blood pressure observations were attempted, however, it might be inferred that such marked loss of kidney substance would result in increased blood pressure and cardiac hypertrophy. Such hypertrophy did not occur in the cat. Joseph⁹ gives the ratio of the heart weight with the body weight in 26 cats as 0.45 per cent, in this laboratory a similar average in 20 cats gave 0.40 per cent. The ratio in the cat under discussion was 0.44 per cent. The dog reported had a fairly large goitre so that similar observations have little value. (However, the ratio was markedly increased—1.44 per cent as compared with 0.743 per cent reported by Joseph.)

⁷ P. B. Hawk, *this Journal*, iv, p. 321, 1908.

⁸ T. C. Janeway, *Soc. of Exp. Biol. and Med.*, vi, p. 109, 1908.

⁹ D. R. Joseph, *Journ. of Exp. Med.*, v, p. 521, 1908.

CONCLUSIONS

Ligation of one of the branches of both renal arteries, *i e*, approximately half of the blood supply, does not cause any noticeable disturbance in renal function. The urine and the nitrogen excretion remain practically normal, with a slight tendency to nitrogen retention, which is probably not accidental.

Successive complete ligation of one renal artery and one branch of the other (*i e*, shutting off three-fourths of the arterial supply) results in marked temporary prostration, anorexia, and loss of weight, with nitrogen output much greater than the intake. The animals recover gradually to a condition similar to that when but one-half of the arterial supply was ligated. On the assumption (justified by MacNider's histological data) that the ligated areas took no part in urine formation, the remaining one-fourth of the kidney was able to secrete the urine almost as effectively as the entire kidney area.

Occlusion of one branch of the renal artery does not result in sufficient collateral circulation from the capsule to preserve the function of the ligated area, so that even after twelve months it is unable to preserve life if the renal artery is then ligated.

With but one-fourth of the kidney substance functioning the quantity of urine was practically normal, the urine contained no albumen or casts, cardiac hypertrophy did not occur.

I take pleasure in acknowledging my indebtedness to Professor Sollmann for his aid during this work.

The total average weight of the normal fetal glands is 9.6 grams with a maximum of 19.8 grams and a minimum of 1.7 grams. The large glands show an average of 40.0 grams, maximum of 194.0 grams and a minimum of 20.2 grams, while the average weight of the adult thyroids is only 36.2 grams.

Before discussing the tabulated results the following data and observations in regard to age, size, weight and sex of the various fetuses and adult animals should be given.

The fetuses, when collected for analysis, were taken at random from the supply at hand at different times. In some lots younger fetuses predominate while in others nearly mature fetuses are in the majority. It was, however, decided to analyze the lots as they came along without any further distinctions than those already described.

As stated above, the range of ages of the fetuses is from six to nine months and this naturally exerts a proportional influence on the size of the fetuses. The several breeds of cattle also differ materially. The weights of all the individual fetuses were not recorded when the thyroid glands were removed, this was due to lack of facilities, but it was noticed that fetuses possessing enlarged glands, although otherwise apparently well proportioned, invariably were smaller than the fetuses with normal glands. This feature was very striking. The fetuses used in this investigation weighed from 20 to 100 pounds.

The possible influence of castration upon the iodine content of the thyroid glands is, of course, eliminated as far as fetuses are concerned, whereas a large percentage of the adult animals used for comparison have been subjected to this operation.

By comparing the figures in the tabulation it will be seen that the amounts of iodine in the normal fetal glands, in spite of their fewer numbers and considerable unevenness in size and age, are much more uniform throughout the entire year than those of the thyroids from full-grown animals and it seems apparent that the seasonal change in iodine content, which is so evident in the healthy glands from mature animals,⁵ is not pronounced in the normal fetal thyroid.

Normal fetal glands are relatively larger and contain more iodine and phosphorus per unit of body weight than thyroids from fully

⁵ Seidell and Fenger *this Journal*, xii, p. 517, 1913

mature animals As would be expected the male glands are somewhat larger than the female thyroids, the average weight being 9.9 grams for the male and 9.3 grams for the female, but it should also be noted that the smaller female glands contain a higher percentage of iodine than the male glands As proportions and sizes of these glands correspond fairly well with the differences in body weight of the two sexes, the suggestion presents itself that the female metabolism requires more thyroid activity according to body weight than the male during the fetal stage as well as in the animal after birth

A most striking feature of the tabulated results is the high percentage of abnormally large fetal glands This enlargement affecting both lobes and the isthmus is quite pronounced at the third or fourth month of intra-uterine life, increasing proportionately with the growth of the fetus It is, therefore, very evident that a large percentage of these animals pass through the fetal stage of life with exceptionally large glands low in iodine content

It is generally understood that a certain number of goitrous glands prevail throughout the year in fully mature cattle, but observations indicate that climatic conditions, at least in the United States, exert a decided influence on the growth and that the enlargement affects a considerably higher number of adult animals during the winter and spring, than in summer and fall The goitrous glands from full-grown cattle, which have come under observation during the last eighteen months, ranged in weight from 150 to 400 grams with average iodine and phosphorus contents of 0.016 per cent and 0.85 per cent respectively These glands were all obtained in the winter and spring months and although beef thyroids in numbers of from 25 to 100 were collected twice a week during all this period of eighteen months, no goitrous glands were encountered in the summer and fall seasons The percentage of goitrous glands in grown animals even during the cold season does not begin to compare with the proportion of enlarged glands found in the fetuses

It should be stated here that the possibility is not entirely eliminated that the pregnant animals whose fetuses show enlarged glands all come from goitrous regions of the country, but if that holds true we naturally should expect to find a similar high percentage of goitrous glands in the general run of adult animals, and this is certainly not the case

It has not been definitely determined or verified whether the enlargement of the thyroid gland, which affects so many of the fetuses, disappears rapidly after birth or persists for some time in the young animals. At present, however, observations indicate that, next to the fetuses, large glands are most frequently met with in sucklings and that there is a gradual decrease in the percentage of affected glands as the animals mature.

The fact that so many fetal glands are disproportionately enlarged, while the adult glands remain undisturbed, makes it very difficult to explain the fetal enlargement simply as a goitrous infection resulting from the ingestion of toxic food or water by the pregnant animal. At the same time the general appearance of these large fetal glands and their low iodine and high phosphorus contents coincide so closely with the corresponding features of goitrous glands from mature animals that the resemblance is very striking. This discrepancy may be explained by concluding that the demand for iodine in the rapid fetal metabolism and growth, in certain instances, may exceed the available supply furnished by the pregnant animal. This supply may be sufficient for the maintenance of the maternal metabolism leaving the adult thyroid normal, but not sufficient to prevent iodine starvation and enlargement of the fetal gland.

Another factor of considerable importance, shown by the tabulated data, is the relative proportion of iodine to that of phosphorus in the various glands. In spite of the size the enlarged glands contain less total iodine and much more total phosphorus than the normal thyroids. This peculiar condition holds true in case of fetal as well as adult glands, and is not specific to beef thyroids only, as it has been identified, for instance, in all goitrous hog and sheep thyroids which have so far come under observation. Since accumulation of organic phosphates does not occur in normal glands of average iodine content, the idea readily suggests itself that iodine in thyroid combination may be a factor of vital importance in the phosphorus metabolism of the body.

It may be mentioned that the work of collecting this raw material is time-consuming and often connected with considerable difficulties, as the fetuses are not plentiful the year round and are almost unobtainable at certain seasons. It is furthermore impossible to obtain systematic information concerning the sources and

localities from which the various animals are derived, thus further masking the evidence and making it more difficult to collate the real facts into a conclusive whole

SUMMARY

It has been demonstrated conclusively that functional therapeutic activity and presence of iodine coincide in the fetal thyroid during intra-uterine life in analogy with the conditions existing during extra-uterine life

The iodine content of the normal-sized fetal thyroid glands during the last three months of intra-uterine life seems to be fairly uniform throughout the various seasons

Normal fetal glands are relatively larger and contain more iodine and phosphorus per unit of body weight than thyroids from fully mature animals

The normal female fetal thyroid glands show a higher content of iodine and seem, therefore, to possess greater functional activity than the male fetal glands

Fetuses possessing enlarged thyroid glands on the whole were considerably smaller than average fetuses of the same age with normal thyroids

The enlargement of fetal thyroids exceeds, both in frequency and size, the number of goitrous-affected glands of fully mature animals and is apparently the consequence of insufficient supply or faulty assimilation of iodine on the part of the pregnant animal

Enlarged glands in general, both fetal and adult, contain less total iodine and much more total phosphorus than normal thyroids

THE UTILIZATION OF AMMONIA IN THE PROTEIN METABOLISM

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It has long been the opinion of the physiological chemist that the higher animal organism was dependent upon the amino-acids for the maintenance of protein anabolism. That plants of all orders, from the lowest to the highest, are able to synthesize protein from the salts of ammonium is, of course, fully established. For the animal organism, however, the prevailing opinion seemed conclusively established to the effect that the building-stones of protein could not be synthesized from ammonia and fatty acids. Formulated in chemical terms, the animal organism was not held to possess the power of effecting the replacement of one hydrogen attached to the α -carbon of a fatty acid by an NH_2 radical to form an α -amino-acid.

Ammonium introduced into the body from without, formed within the tissues through reactions of metabolism, or formed within the intestinal tract by the action of bacteria, was held to be converted into urea or eliminated as such. It was not held to be converted into amino-acids.

Recently published experiments by Knoop,¹ Embden² and their confrères have brought evidence to the effect that the animal body does possess the power of converting aliphatic and aromatic α -ketonic acids into amino-acids. This has led Grafe and Schlapfer³

¹ Knoop *Zeitschr f physiol Chem*, lxxvii, p 489, 1910

² Embden *Biochem Zeitschr*, xxix, p 423, 1910

³ Grafe and Schlapfer Über Stickstoffretention und Stickstoffgleichgewicht bei Fütterung von Ammoniumsalzen, *Zeitschr f physiol Chem*, lxxvii, p 1, 1912

and Abderhalden⁴ to study the effect of ammonia on the protein metabolism of dogs. Grafe and Schlapfer came to the conclusion that dogs on a high carbohydrate diet, with low protein intake, were able to retain very considerable quantities of the N of ammonia (NH_4Cl). With larger quantities of ammonium citrate, they were able to maintain nitrogenous equilibrium for a period of fifteen days. They did not notice any elimination of retained nitrogen during the after periods. Grafe and Schlapfer, therefore, reached the conclusion that the animal has the power of synthesizing protein from carbohydrate and ammonium. Abderhalden's method was similar to Grafe's. His first dog received, during the fore-period of eight days, 40 grams of fat, 40 grams of sugar, 40 grams of starch and 5 grams of bone ash, per day. The nitrogen balance for that period, as was to be expected, was strongly negative (-1.83 to -2.95 per day). During the following three days, ammonium carbonate was added to the diet, and the nitrogen balance was -0.23 , -0.08 , -0.09 gram per day. During the after period, the nitrogen elimination was not any higher than during the fore-period. Experiments with ammonium acetate did not show such marked retention, but showed a very considerable retention. Abderhalden hesitatingly reached the conclusion that "the addition of ammonium salts as the only source of nitrogen to a diet consisting of carbohydrates and fats, exercises a distinct influence on the protein metabolism, by causing nitrogen retention." The relationship of this retention to the protein anabolism, he left an open question, suggesting, however, the probability that a reversible reaction had set in.

In later work on the subject,⁵ Abderhalden tends to incline more to the retention hypothesis, denying the possibility of the synthesis of protein from ammonia and carbohydrates. He writes "It is no doubt possible in some cases to reduce the nitrogen elimination by ammonium salts. We have not observed any

⁴ E. Abderhalden, Fütterungsversuche mit vollständig bis zu Aminosäuren abgebautem Eiweiss und mit Ammonsalzen, *Zeitschr. f. physiol. Chem.*, lxxviii, p. 1, 1912.

⁵ E. Abderhalden and Paul Hirsch, Fortgesetzte Untersuchungen über die synthetischen Fähigkeiten der tierischen Zelle. Versuche über die Verwertung verschiedener Stickstoffquellen im Organismus des Hundes, *Zeitschr. f. physiol. Chem.*, lxxxii, p. 1, 1912.

prolonged positive nitrogen balance. Only in a few cases was it temporarily positive. It was, however, more negative in the after period. We are inclined to assume that the established nitrogen does not stand in any direct relation to the synthesis of protein. We have no reason for the assumption that the animal organism has the power of producing the different complexes for the variously built amino-acids from carbohydrates, and that the administered ammonia is used for their aminization. Such an assumption stands in contradiction to all the present conceptions of protein metabolism."

On examining Abderhalden's figures, one fails to notice any marked "Ausschwemmung" of the retained nitrogen in the after periods.

The experiments reported below were performed with the object of throwing light on several questions which presented themselves to us on reviewing the subject.

1 *Does the ammonia combine with some rest of the carbohydrate molecule in its retention, is the carbohydrate really necessary for the demonstration of the utilization of ammonia in the protein metabolism, and does the carbohydrate give rise to the non-nitrogenous fraction of the various amino-acids?*

Grafe maintains throughout his studies that large quantities of carbohydrates are necessary to get a satisfactory demonstration of the utilization of ammonia, and Abderhalden has accordingly kept his animals on a high caloric intake. The results of Grafe's and Abderhalden's experiments are very similar. The differences are only in the degree of retention. Grafe maintains that the differences in their results are to be sought in the difference of the carbohydrate supply, and that the quantity of the carbohydrate fed is the determining factor.

It seems to us that the understanding of the state of retention of the nitrogen involves this question directly. Is the carbohydrate a very important factor? Does the circulation of an excess of carbohydrate molecules in the blood bring about the synthesis with the ammonia to amino-acids, which either build up or spare body protein, or does the carbohydrate play a secondary rôle, and the ammonia the primary rôle?

We have attempted to solve this problem by feeding ammonium carbonate to starving dogs. If the carbohydrate is of importance,

and if the ammonia in its retention is tied up with the carbohydrate molecule, the results should be negative. That is, none of the ammonia nitrogen should be retained. On the other hand, if on giving ammonium carbonate, a retention of the nitrogen should occur, Grafe's "carbohydrate" theory can safely be eliminated.

Dog 1 was permitted to fast for three days prior to the commencement of the experiment. Water was given *ad libitum*. The animal was kept in a metabolism cage, and the urine collected quantitatively. The periods of twelve hours were separated by catheterizing and washing the bladder with distilled water. The nitrogen was determined according to Kjeldahl, the ammonia according to Folin. The ammonium carbonate was prepared by Baker, and has the following composition $(\text{NH}_4)_2\text{CO}_3 \cdot \text{NH}_4\text{CO}_2\text{-NH}_3$.

EXPERIMENT I
Twelve-hour periods

PERIOD	WEIGHT	TOTAL NITROGEN	NH ₃ N	NITROGEN BALANCE	REMARKS
I	8 32	1 01	0 058	-1 01	{ 1 gram of N in the form of ammonium carbonate given <i>per os</i>
II		0 88	0 065	-0 88	
III		0 89	0 052	-0 89	
IV	8 05	1 28	0 076	-0 28	
V		0 87	0 054	-0 87	

This experiment shows very clearly that a good part of the nitrogen (more than half a gram) was retained, and was not eliminated in the after period.

To avoid the criticism of too short periods, the periods of the next experiments were extended to twenty-four hours.

EXPERIMENT II
Twenty-four-hour periods

PERIOD	WEIGHT	TOTAL NITROGEN	NH ₃ N	NITROGEN BALANCE	REMARKS
I	17 68	3 26	0 24	-3 26	{ Sixth fasting day 2 grams of N in the form of ammonium carbonate given <i>per os</i>
II		4 14	0 236	-2 14	
III	17 32	3 22	0 403	-3 22	

The results of this experiment corroborate the findings of the first. Both agree in showing that a considerable part of the nitrogen of the ammonia is retained, and that it is not eliminated in the after period.

EXPERIMENT III
Twenty-four-hour periods

PERIOD	WEIGHT	TOTAL NITROGEN	NITROGEN BALANCE	REMARKS
I		2 43	-2 43	Eighth fasting day
II		2 32	-2 32	
III		2 39	-2 39	
IV		2 21	-2 21	
V	8 95	2 13	-2 13	
VI	8 85	1 96	-1 96	
VII	8 79	2 00	-2 00	
VIII	8 69	2 45	-2 45	
IX	8 74	2 16	-2 16	
X	8 66	3 12	-0 82	73 grams of meat = 2 3 grams of N
XI	8 53	1 90	-1 90	
XII	8 48	1 75	-1 75	
XIII	8 44	2 13	+0 17	2 3 grams of N in the form of meat
XIV	8 36	1 81	-1 81	
XV	8 20	1 62	-1 62	
XVI		2 74	-0 44	{ 2 3 grams of N in the form of ammonium carbonate <i>per os</i>
XVII		1 83	-1 83	
XVIII	7 80	1 72	-1 72	
XIX		3 40	-1 10	{ 2 3 grams of N in form of ammonium carbonate given <i>subcutaneously</i>
XX		2 91	-2 91	
XXI		2 37	-2 37	
XXII	7 48	3 97	-1 67	{ 2 3 grams of N in the form of urea given <i>per os</i>
XXIII	7 32	2 00	-2 00	
XXIV		1 76	-1 76	
XXV	6 98	4 02	-1 72	2 3 grams of N in the form of urea
XXVI	6 95	2 16	-2 16	

This experiment, in addition to the fact that it corroborates the first two, also shows that the nitrogen of ammonia may affect the nitrogen balance almost to the same extent as does the feeding of meat. Ammonia administered subcutaneously is eliminated quantitatively. Urea administered *per os* is eliminated quantitatively.

The outcome of these experiments proves very conclusively that the nitrogen of ammonia can be retained in the system even in the absence of carbohydrates, and Grafe's theory can therefore be discarded. The ammonia is undoubtedly utilized in some other way. That it is not retained as an end product, urea, we believe to be demonstrated by the fact that a corresponding dose of urea is completely eliminated within twenty-four hours.

The second question that presented itself was *Will ammonia nitrogen be retained in the case of glucosuria, where the utilization of carbohydrates is almost completely lost?*

Two experiments were performed on completely phlorhizinized dogs. The results are as follows:

EXPERIMENT IV

Twelve-hour periods

PERIOD	TOTAL NITROGEN	GLUCOSE	D N	NH ₃ N	REMARKS
I	8.20				2 grams of NH ₃ N
II	8.06				
III	8.30				

EXPERIMENT V

I	6.65	25.93	3.89	0.35	3 grams of N as ammonia
II	6.66	25.88	3.87	0.46	
III	7.40	25.35	3.42	0.32	
IV	6.21				

These two experiments show that a diabetic animal has the power of retaining ammonia nitrogen to a much larger extent than does the starving animal.

How can we explain these peculiar retentions of nitrogen? Can we bring them into harmony with known facts, or must we seek some other interpretation?

Abderhalden's latest assumption, that the nitrogen is retained temporarily, is not borne out by his own figures. The fact that there happened to be an increase in the nitrogen elimination on a starvation day immediately following the ammonia feeding, is not sufficient to prove the point. It merely illustrates the necessity of carefully separating the urine at the end of each twenty-four hours, which both Abderhalden and Grafe seemed to consider

unnecessary, and which makes the interpretation of their results considerably more difficult. Grafe's theory that the carbohydrates pass into union with the nitrogen of the ammonia is here proven to be erroneous. The most probable answer to the above questions, and the one most strongly in harmony with well-known physiological and chemical facts, is the following: the ammonia, because of its high concentration in the tissues, most probably in the intestinal wall and liver, reverses the process of deamination. By catabolism of protein in the cells, we understand a cleavage of protein into the constituent amino-acids that make up the protein molecule. These amino-acids are believed to be deaminized, the amino radical going into urea, and the non-nitrogenous fraction either broken down and burnt directly or utilized in the synthesis of glucose, glycogen and fat. It is at the point of this deamination that we believe the ammonia to exert its influence. It is now well established that the non-nitrogenous part of the deaminized amino-acids are either α -hydroxy or α -ketonic acids, both of which when administered into the body or perfused through the liver have the power of combining with the amino radical.

We have attempted to test this theory in the following way. Grafe, Abderhalden and the writers, in the experiments reported above, administered all the ammonium carbonate in a rather concentrated form. This causes the ammonia to be absorbed rapidly, and to exert a high mass action. It was thought that, by giving the ammonia in very high dilution and in small quantities at a time, the mass action might be eliminated.

The subject of this experiment was a man of about 70 kilos body weight, in medium flesh. The diet consisted of 200 grams of starch, 200 grams of cane sugar, 5 grams of table salt and 3000 cc of water. The starch was the best of several preparations, the one used contained only 0.065 gram of nitrogen in the day's ration. The diet could fairly be termed nitrogen-free. It contained 1600 calories, not enough to place the individual in caloric equilibrium, but enough to spare body protein effectively. The water was consumed with the food, three times daily. The subject was placed on this diet for a fore-period of five days. The urine and feces were collected only during the last three days of the fore-period. The urine was analyzed for total nitrogen by the method of Kjeldahl, ammonia and creatinine by the methods of Folin, urea

by the latest method of Benedict, and the purine bodies by successive precipitation with silver nitrate and copper bisulphate, the final precipitates being analyzed for their nitrogen content by the method of Kjeldahl. The feces were dried in the usual way.

On the sixth day ammonium carbonate was administered in three doses with the food in dilute solution, the salt being dissolved in the water taken with the meal (750 cc). The ingestion for the day was 11.37 grams, containing (by analysis) 2.8 grams of nitrogen. On the following day, 11.6 grams of ammonium carbonate were ingested in a similar manner, containing 2.86 grams of nitrogen. The two following days constituted the after period, the diet being maintained.

Such doses of ammonium carbonate are very irritating to the intestinal tract. Diarrhea set in, so that a marking of the stools became impossible. The feces of the entire period were collected, therefore, and analyzed as one. This introduced an error, or at least an opportunity for error, since the degree of resorption of ammonium carbonate could not be definitely fixed. As will be later detailed, no error of consequence was committed, as the nitrogen content of the feces was not abnormally high. We had hoped to extend the after period, since we wished this period to be so long as to absolutely exclude any later elimination of retained nitrogen. However, when the low figure for the nitrogen output for that day became known, the experiment was discontinued, largely on account of the intestinal distress, which made it advisable to seek another diet. Other symptoms of the action of ammonium than those described were not noted, in particular, such high doses had no effect upon the circulation.

The following table contains the analytical data of the experiment. All values are expressed in grams.

EXPERIMENT VI

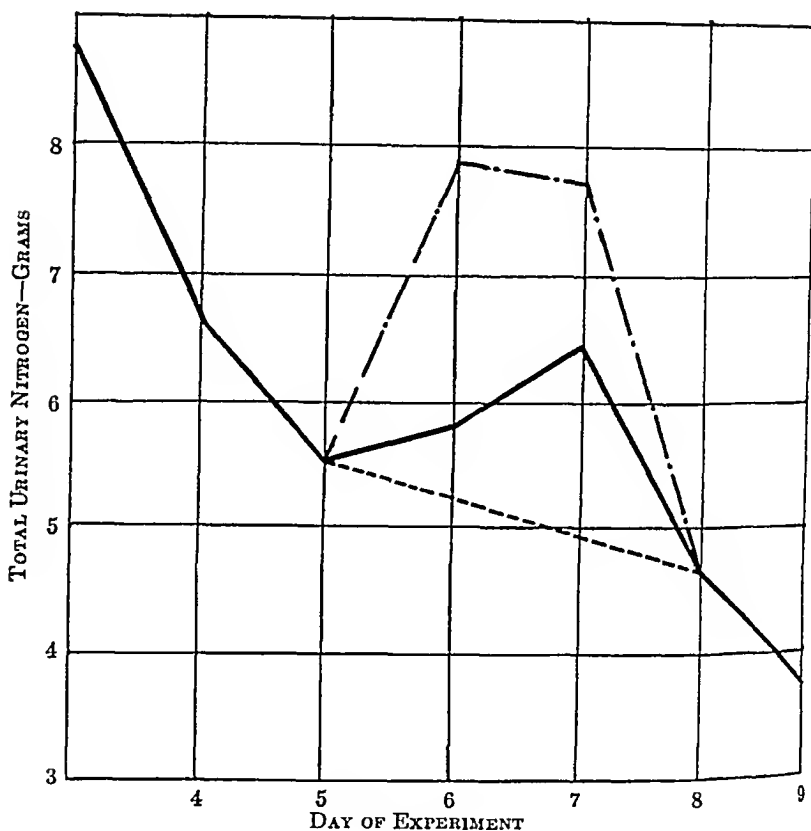
DAY OF EXPERIMENT	3	4	5	6	7	8	9
Urinary N	8.86	6.64	5.54	5.84	6.39	4.66	3.75
Fecal N	0.76	0.76	0.76	0.76	0.76	0.76	0.76
Total N output	9.62	7.40	6.30	6.60	7.15	5.42	4.51
Urea N	6.58	4.07	3.47	3.62	4.51	2.27	1.81
Ammonia N	0.54	0.73	0.41	0.42	0.41	0.37	0.41
Creatinine N	0.64	0.66	0.68	0.66	0.64	0.67	0.66
Purine N	0.050	0.045	0.045	0.045	0.070	0.055	0.040
Rest N	1.05	1.13	0.94	1.10	0.70	1.5	0.83

The average fecal nitrogen, 0.76 per day, cannot be considered in any way above the normal. Obviously, the ammonium carbonate was fully resorbed. And the diarrhea made apparently no difference in this resorption. If there was any effect, there was possibly an increase in the secretory nitrogen eliminated, though, as stated, 0.76 gram of nitrogen per day cannot be considered high, it is within the usual limits for normal figures.

Strikingly constant are the figures for creatinine and purine and, with the exception of one day, of ammonia also. There is a short rise in the purine on one day, the second day of the test, leading possibly to the inference of increased nuclear catabolism in the liver, as the result of, or associated with, the presence of the excess of ammonium carbonate in the liver.

From an inspection of the figures for the total urinary nitrogen and urea, it is clear that there was a rise, associated with the ingestion of the ammonium carbonate, but which was not at all commensurate with the input. The greatest part of the administered ammonia was retained in the system, and was not eliminated in the after period. This shows that the ammonia is retained even when given in dilute form. It does not, however, vitiate the theory of the reversible action of ammonia on deamination. For dilute as the ammonia was when administered, we have no means of telling at what rate and in what concentration it was absorbed. We hope to test this again by dividing the ammonia into very small doses, administered at very short intervals during the day.

The curve yields a graphic reproduction of the data. The heavy continuous line represents the actual figures for urinary nitrogen. The dotted curve for the two days indicates approximately what would have been the curve of elimination had no ammonium carbonate been administered. The dashed line indicates what would have been the curve of elimination had all the nitrogen of the ingested ammonia been eliminated. It is clear that the larger fraction of the nitrogen has been retained.



SUMMARY

Three experiments were performed on starving dogs, and the nitrogen output studied. Ammonium carbonate was given *per os*, and it was found that a considerable part of the nitrogen was retained and failed to be eliminated in the after period. When given subcutaneously, it was promptly eliminated.

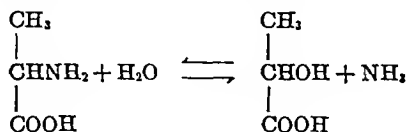
The administration of urea was followed by a complete elimination of all the nitrogen.

Ammonia administered to diabetic dogs was also retained, but to a larger extent than in normal dogs.

Ammonia given to man on a protein-free diet (0.065 gram per day) was retained to the extent of about two-thirds.

These experiments corroborate the findings of Grafe and Abderhalden, and also show that the presence of carbohydrates is not an obligatory factor in the retention of nitrogen from ammonia

It is suggested that the ammonia nitrogen is retained because of a reversed reaction that leads to combination with the α -ketonic or α -hydroxy-acids to form amino-acids which may be used in the upbuilding or sparing of body protein. This may be illustrated in the simple reaction for the deamination of alanine



Like every reaction, this reaction must be reversible under appropriate conditions. What the station of equilibrium in the animal body may be, we have no way of knowing. But according to the interpretation of the retention that we are inclined to accept, with the administration of large amounts of ammonia this reaction is reversed, probably in the liver. Whether such a situation as this might arise outside of the experiment, normally or pathologically—the formation of such amounts of ammonia as to reverse the reaction—is problematical. It is possible that such concentration of ammonia within the portal system could never occur naturally, in health or disease. In that event, this experiment would simply demonstrate the possibility of reversion of reaction in the animal body and add another illustration to the long list of demonstrations of the validity of physico-chemical laws within living bodies. We have used alanine as the simplest illustration of the reaction, and in view of the omnipresence of lactic acid within the organism, it is possible that the retention occurs largely or entirely in this state. The body could then make use of this alanine to the same extent and in the same way that alanine derived from the hydrolysis of protein is utilized.

If one does not incline to the view that the ammonia is retained in combination, one must assume that it is retained either in the state of ammonia or urea. The latter appears unlikely, in view of the prompt and complete elimination of urea in the direct experiment. The retention of ammonia as such, in the form of a salt, appears to us quite unlikely.

Since the completion of this paper one more experiment upon a dog was performed by Dr L Jonas of this department, which corroborates our previous findings

EXPERIMENT VII
Twenty-four-hour periods

PERIOD	TOTAL N	N BALANCE	REMARKS
I	3 59	-3 59	1 04 grams N as ammonium carbonate
II	4 04	-4 04	
III	3 64	-3 64	
IV	3 52	-3 52	
V	3 86	-2 81	
VI	3 54	-3 54	
VII	3 15	-3 15	

STUDIES IN THE PURINE METABOLISM

I ON URICOLYSIS IN THE HUMAN SUBJECT

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The purine bases are, according to almost universal belief, converted into uric acid or eliminated unchanged, this applying to both endogenous and exogenous purine bases. To what extent the elimination of uric acid, however, is to be regarded as expressive of the formation of uric acid in catabolism (the factor of circulatory retention being disregarded) is, despite much investigation, not yet clear. The most current opinion runs to the effect that a relatively large amount of the uric acid formed in metabolism, or ingested, is converted into urea under the influence of uricolytic ferments, such as have been isolated from the organs of lower animals. Without at this time entering into a discussion of the literature, we wish to report a carefully controlled experiment on the human subject. Experiments to be reported in a subsequent paper deal with the influence of the input of nitrogen and of glandular work upon the elimination of uric acid.

The subject of the experiment was a healthy young adult, a student of medicine, weighing about 69 kilos. The test extended over fourteen days. Wishing to exclude rigidly any possible influence of the input of nitrogen and of glandular work, the diet was constant in nitrogen and in calories during the entire period. The plan of the test was to place the subject for three days upon a diet of milk, egg, starch and sugar. During the following three days, a fraction of the milk-egg was substituted by sweetbreads containing the same amount of nitrogen. During the next four days this substitution was doubled. During the last four days the subject was again placed upon the diet of the first period. The nitrogen during the four periods was exactly 10 grams per

day The caloric value of the diet was 2000 calories per day The diet of the first period was prepared daily as follows Four eggs were beaten into two liters of milk, and the nitrogen determined by the method of Kjeldahl An amount of this mixture containing 10 grams of nitrogen was then measured off, and enough sugar and nitrogen-free starch added to bring the caloric value to the determined plane The food was then cooked in the form of a custard, and ingested in three meals This continued as stated for three days Upon the fourth day, an amount of the mixture of milk and egg corresponding to 3 grams of nitrogen was withdrawn, and replaced by a portion of sweetbreads containing 3 grams of nitrogen The sweetbreads were ground in a fine mill, and cooked in the form of a loaf, in which state duplicating estimations of nitrogen could be secured The caloric input remained unchanged After four days of this regimen, 3 further grams of the milk-egg nitrogen input were substituted by an equal amount of nitrogen in the form of the sweetbreads, the caloric input being unchanged During the last four days the subject was returned to the diet of 10 grams of nitrogen in the form of milk and egg Thus the nitrogen input of the tests was as follows

	grams
<i>First period</i> milk-egg nitrogen	10
<i>Second period</i> milk-egg nitrogen, 7 grams, sweetbread nitrogen	3
<i>Third period</i> milk-egg nitrogen, 4 grams, sweetbread nitrogen	6
<i>Fourth period</i> milk-egg nitrogen	10

It was assumed that the digestion and assimilation of 1 gram of sweetbread nitrogen corresponded with that of 1 gram of milk-egg nitrogen In the first and fourth periods the subject was on a purine-free diet, in the second and third periods, the subject was upon a known and controlled input of exogenous purine in the shape of nucleic acids of the sweetbreads The feces were marked and collected The urine was collected in periods, not in days, in order to avoid the fluctuations of daily eliminations The figures in the tables represent therefore the daily averages of each period, and the evenness of these figures is of course due to this procedure The urine was analyzed for total nitrogen by the method of Kjeldahl, for urea by the method of Benedict, for ammonia and creatinine by the methods of Folin, for uric acid by

the new colorimetric method of Fohn, the dried feces by the method of Kjeldahl. The purine bases were estimated as follows

Six hundred cc of urine are freed of phosphates by the addition of 50 cc of magnesia mixture, 50 cc of 5 per cent ammonia added, filtered and of the filtrate 600 cc taken for the estimation of purine. The purine bodies are first precipitated according to the method of Salkowski with silver nitrate. The precipitate is collected, washed free of chlorides, suspended in water, a drop of hydrochloric acid added, the silver precipitated with hydrogen sulphide, heated, filtered hot and the filtrate evaporated to dryness at about 70°. Important in the operation is a clear filtrate after precipitation of silver sulphide, quick filtration of the hot solution, and certainty that at all times the reaction of this filtrate is acid. The dried residue is then taken up in hot water, the smallest traces of sodium carbonate added until the uric acid passes entirely into solution, the hot solution faintly acidulated with acetic acid and filtered at once into a marked 250 cc flask, the filter paper washed with hot water, the flask filled to the mark with hot water, and then two portions of 100 cc each measured into beakers, and the purines precipitated with copper sulphate and sodium bisulphite according to the method of Kruger. The washed precipitates are finally transferred to Kjeldahl flasks and the nitrogen estimated. To determine the purine nitrogen of the total urine the obvious calculations must be carried out. To determine the nitrogen of the purine bases, the nitrogen of the uric acid determined apart must be subtracted. This method has been applied to urines to which known additions of purine bases and of uric acid have been made, with checking results. The new Fohn method for the estimation of uric acid has been found to check well with the results of the method of Salkowski, and is carried out in a twentieth of the time.

The following table presents, in terms of nitrogen daily, the results of the experiment. All figures are rounded.

DAYS	1 2 3	4 5 6	7 8 9 10	11 12 13 14
Total urinary N	8.9	8.7	9.1	8.8
Urea N (+ NH ₃)	7.3	7.1	7.1	7.05
Creatinine N	0.58	0.55	0.56	0.47
Purine N	0.11	0.17	0.26	0.10
Uric acid N	0.09	0.14	0.24	0.07
Rest N	0.91	0.88	1.18	1.18
N input	10.0	10.0	10.0	10.0
Purine N input	0	0.17	0.34	0
Fecal N output,				
Average	0.5	0.5	0.5	0.5

The figure given for the purine nitrogen of the sweetbreads is approximate only, it is certainly too low. The nucleic acids were freed according to Neumann, precipitated with alcohol, then hydrolyzed with hydrochloric acid, then estimated just as described for purine nitrogen in the urine. The stools contained but mere traces of nucleic acid in the cell- and bacteria-free filtrate, apparently, judging by this and by the nitrogen of the stools, the digestion of the sweetbreads was normal and complete.

From these figures it is clear that although the replacement of milk-egg nitrogen by sweetbread nitrogen results in a rise in the purine nitrogen of the urine, an expression of the elimination of purines derived from the catabolism of the nucleic acids of the sweetbreads, this increment in elimination is less than half of the known input of purine in the state of nucleic acids in the sweetbreads. The proportional rise in the elimination during the third period as contrasted with the figure for the second period, must have been purely accidental. It is clear from these figures that the larger portion of the ingested purine was either destroyed in the alimentary tract prior to resorption, or was converted in the metabolism into non-purine (presumably into urea), since less than half of the ingested amount was recovered in the urine. It is also clear from the figures that the ingestion of a moderate amount of purine bases does not lead to increase in the purine bases in the urine, but solely to increase in uric acid.

ON GLYOXALASE

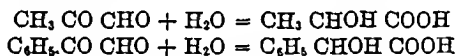
BY H D DAKIN AND H W DUDLEY

(From the Herter Laboratory, New York)

(Received for publication, April 2, 1913)

In a recent communication¹ the writers have given a preliminary account of the formation of α -hydroxy-acids from α -ketonic aldehydes as the result of ferment action. The present paper is concerned with a more detailed study of the reaction.

The catalyst with which we are concerned effects the rapid conversion of methyl glyoxal into lactic acid and of phenyl glyoxal into mandelic acid—a reaction which is readily imitated *in vitro* by the action of alkalis



It is probable that other glyoxals may undergo similar changes but, from a biochemical standpoint, the formation of lactic acid from methyl glyoxal is of particular interest on account of the relation of the latter substance to the carbohydrates.

We shall present evidence indicating the enzyme character of the catalyst in question and, in conformity with current nomenclature, we propose that it be named "glyoxalase."

We find that a solution exhibiting strong catalytic activity, such as may be made by straining an aqueous extract of muscle or liver from an exsanguinated dog or rabbit, may be heated for a short time to 48° without much injury but at once loses its activity on heating to 60° . On allowing the tissue extract to stand at room temperature, without addition of antiseptics, the activity is lost relatively slowly. After forty-eight hours there is not much diminution in activity, but after four or five days the solution contains but little of the enzyme.

¹ This *Journal*, xiv, p 155, 1913

Experiments aiming at a rough purification of the catalyst by precipitating filtered tissue extracts with alcohol were unsuccessful, but by precipitating with solid ammonium sulphate and dialyzing the suspension of the precipitate in water, an active solution of the enzyme was obtained

In order to obtain some information as to the conditions under which the enzyme is active, we have made experiments as to the effect of the presence of acid and alkali. We find that, above a certain small concentration, acids such as acetic acid (0.1 per cent) exert not only an inhibitory effect, but actually destroy the enzyme so that no action is observable on subsequent neutralization. Moderate amounts of alkali (0.1 per cent Na_2CO_3), on the other hand, have no such action. The inhibitory effect of acids would seem to be of importance, for it obviously indicates a regulatory mechanism for the action of the enzyme, for both methyl glyoxal and the glucose which may yield it are substantially neutral, while an accumulation of the product of the enzyme action, lactic acid, would automatically inhibit further acid formation. It appears likely that we have to do with a delicately adjusted equilibrium which may roughly be represented as follows



Additional support as to the possibility of some such series of changes occurring in the animal body is furnished by the fact that we have recently been able to demonstrate the reversibility of the methyl glyoxal lactic acid transformation by experiments *in vitro*. In addition, by the action of sodium phosphate upon glucose, we have been able to obtain a substance which yields hydrazine derivatives of methyl glyoxal and which is therefore either methyl glyoxal or some closely related substance. These experiments, and others upon the fate of methyl glyoxal in the diabetic organism, will be reported in future communications.

The fact that acid, whether formed by the action of the enzyme or added to the solution, inhibits the reaction, makes it necessary to provide for the automatic neutralization of the solution when observing the action of the enzyme. Indeed the speed of lactic acid formation is so great when moderate amounts of tissue extract are employed that it depends substantially upon the rate of neutralization. If neutral methyl glyoxal solution is added to a neutral

tissue extract and the mixture incubated at 37°, it is noticed that after a few minutes the solution becomes acid and a heavy precipitate of proteins is formed. The acidity may increase until it is about equal to a $\frac{N}{100}$ solution of lactic acid (0.09 per cent) and then no further formation of acid occurs. For purposes of neutralization we have employed sodium bicarbonate and sodium phosphate and freshly precipitated calcium carbonate. The latter appears to be preferable. The disadvantage of the phosphate is that such large amounts are necessary to neutralize relatively small quantities of lactic acid and, while the action of the carbonate is slower, it has little or no action upon the glyoxals and it is not possible for excessive alkalinity to develop.

One of the most curious facts connected with glyoxalase is that its action on both methyl glyoxal and phenyl glyoxal does not yield a single optically active form of the corresponding hydroxy-acid, but a mixture of both forms in unequal proportions. Thus methyl and phenyl glyoxal give a mixture of laevo and inactive lactic and mandelic acids. At first we were inclined to ascribe the formation of the inactive acids to hydrolysis taking place independently of the enzyme, but further investigation does not bear out this supposition, and at present we are inclined to the belief that both dextro and laevo acids are formed as the result of enzyme activity. The evidence for this is based upon the following facts. Firstly Both forms are produced under conditions unfavorable to hydrolysis except by enzyme action, *e g*, in faintly acid solution. Secondly The acids obtained show variations in the proportions of laevo and dextro forms. In fact, in the case of one experiment, an enzyme solution which at first gave chiefly laevo mandelic acid when acting on phenyl glyoxal, on standing and subsequently acting on fresh phenyl glyoxal, gave mandelic acid containing an excess of the dextro acid. This effect was certainly not due to bacterial action, since on further standing the enzyme was destroyed and no appreciable amount of mandelic acid could be obtained.

Judging by analogy, it seems unlikely that a single enzyme would effect an incomplete asymmetric synthesis, such as the conversion of methyl and phenyl glyoxals into mixtures of the laevo and dextro lactic and mandelic acids. So far as the facts are at present available, it would seem probable that more than one enzyme is concerned in the observed reactions.

It is certainly surprising that glyoxalase when acting upon methyl glyoxal should produce an excess of the laevo lactic acid, whereas the dextro acid is the variety ordinarily found in animal tissues. Levene and Meyer² have recently shown that dextro lactic acid is exclusively produced by the action of leucocytes upon glucose and, simultaneously with ourselves, revived Nef's view that methyl glyoxal is an intermediate step in the conversion of glucose into lactic acid. What rôle, if any, laevo lactic acid plays in animal metabolism must be the subject of future investigation, for it is clear that the stereochemical relations are by no means simple. It may be noted, however, that the occurrence of inactive lactic acid in animal tissues has, in the past, been asserted, although Moriya³ believed on the basis of rather inadequate experiments that these statements were inaccurate.

An idea of the striking activity of glyoxalase may be gained from the fact that 100 cc. of an extract prepared by shaking liver tissue with five parts of saline, containing less than a gram of organic matter, is able to convert more than 2 grams of methyl glyoxal into lactic acid in less than fifteen minutes if provision is made for the prompt neutralization of the acid as formed.

It would seem likely that glyoxalase may have a wide distribution, and we have already observed its presence in yeast and in the oyster. Certain lactic acid bacilli which were tested, gave a negative result possibly on account of the failure of the enzyme, if present, to leave the intact cells. We find that the action of glyoxalase observed in blood is confined entirely to the cells, the serum being inactive.

EXPERIMENTAL

Preparation of glyoxalase extract from animal tissues

The animal (dog or rabbit) was bled and washed out with physiological saline under ether anaesthesia. Immediately after death the tissues to be used were removed, finely minced and stirred up with warm (35°C) saline for a few minutes. To each 100 cc. of saline were added 20 grams of minced tissue. The liquid was then strained through muslin. In most experiments the turbid extract

² *This Journal*, xiv, p. 149, 1913

³ *Zeitschr. f. physiol. Chem.*, xliii, p. 397

was used directly, but it may be filtered through paper without losing its activity. The filtrate may also be precipitated with solid ammonium sulphate and on dissolving the precipitate in water and dialyzing an active solution of the enzyme may be obtained.

Investigation of the action of glyoxalase extract on phenyl and methyl glyoxal

a Phenyl glyoxal To a known volume of extract was added a solution of phenyl glyoxal and the substance used for controlling the acidity of the mixture—sodium phosphate, sodium carbonate or chalk. After incubation for several hours at 37°C in the presence of a small amount of toluene solid ammonium sulphate—roughly 50 grams to 100 cc—was added and the liquid was heated in a water bath for ten to fifteen minutes. The ammonium sulphate precipitates any unchanged phenyl glyoxal. The mixture was then allowed to cool, acidified with a few cubic centimeters of phosphoric acid, and, after effervescence had subsided, filtered off from precipitated protein which was washed with ammonium sulphate solution. The filtrate, to which a further small quantity of phosphoric acid was added, was then extracted three times with small amounts of ether, each extract being washed with about 5 cc of water. The residue, after evaporation of the ether, was dissolved in water (10 cc), filtered, examined polarimetrically in a 2 dm tube, and subsequently its acidity determined by titration of 5 cc of the solution with decinormal sodium hydrate.

b Methyl glyoxal In this case the incubation mixture was prepared as for phenyl glyoxal, but for the extraction of the lactic acid produced the following method was first adopted. The protein was precipitated with ammonium sulphate and filtered off as before, and the filtrate, acidified with phosphoric acid, was extracted with ether for many hours. The residue from the ether extract was taken up in water, examined in the polarimeter, and an aliquot part was titrated with decinormal alkali to determine the acidity. The remainder was converted into the zinc salt for identification and analysis.

In later experiments the incubated mixture was faintly acidified with acetic acid and then evaporated nearly to dryness on the water

a Observed rotation, -0.07° 1 cc required 5.8 cc alkali equivalent to 0.6264 gram lactic acid

b Observed rotation, 0° 1 cc required 0.1 cc alkali

c Observed rotation, $+0.02^{\circ}$ 1 cc required 0.3 cc alkali

d 100 cc 20 per cent extract (liver and skeletal muscles of rabbit) + 10 cc chalk suspension + 1 gram methyl glyoxal in 40 cc of water Incubated 18 hours at 37°C

Worked up lactic acid with gypsum as described and extracted in Soxhlet apparatus with ether

Ether extract made up to 10 cc Rotation in 2 dm tube, -0.2° 1 cc required 10.1 cc alkali = 0.909 gram lactic acid

V Inhibition of activity of enzyme due to the production of lactic acid

100 cc of 20 per cent extract (liver and skeletal muscles of rabbit) were mixed with 1 gram of methyl glyoxal in 40 cc of water and incubated at 37°C for 18 hours The precipitate of protein produced was filtered off in the cold 55 cc of the solution were titrated with decinormal sodium hydrate and required 5.2 cc This corresponds to a 0.035 per cent solution of lactic acid, and at this acidity glyoxalase ceases to convert methyl glyoxal into lactic acid

The lactic acid obtained from different experiments was all worked up to zinc lactate This was recrystallized and analyzed

Crop I 0.2944 gram lost 0.0492 gram H_2O at 120°C = 16.7 per cent

Crop II 0.2749 gram lost 0.0386 gram H_2O at 120°C = 14.03 per cent

0.2541 gram zinc lactate (anhydrous) gave on ignition 0.0353 gram ZnO = 33.56 per cent ZnO Theory requires 33.4 per cent

Crop I 0.3052 gram of the recrystallized salt in 10 cc aqueous solution gave a rotation of $+0.32^{\circ}$ in a 2 dm tube

$$[\alpha]_D = +6.36^{\circ}$$

Crop II 0.2749 gram recrystallized salt in 10 cc solution gave a rotation of $+0.42^{\circ}$

$$[\alpha]_D = +8.89^{\circ}$$

Presence of glyoxalase in blood cells

The serum and cells of defibrinated dog's blood were separated by means of the centrifuge After the cells had been washed several times with saline, they were added to solutions containing chalk and phenyl glyoxal In one series of experiments, 25 cc of blood serum were added to a solution of 0.1 gram phenyl glyoxal in 25 cc of water, and blood cells made up to 50 cc with saline were added to a similar solution These were incubated at 37°C in the presence of toluene for twenty hours The mandelic acid

was worked up as usual and the solutions (10 cc) examined in a 2 dm tube gave readings of 0° and -0.45° , respectively. Glyoxalase was evidently absent from the serum but present in the cells.

Using chalk to neutralize the acid formed, it has been possible to obtain large rotations, using the cells from only 5 cc of blood.

Presence of glyoxalase in other organisms

Yeast A mixture of 5 grams of pressed yeast in 50 cc of water with 0.2 gram phenyl glyoxal and 5 cc of chalk suspension was incubated for 24 hours at 37°C in presence of toluene. The mandelic acid was worked up in the usual manner and was readily obtained in crystalline form. The rotation of the solution (10 cc) in a 2 dm tube was -0.42° . Glyoxalase was evidently present.

Oyster 25 grams of the minced tissues were added to a solution of 0.2 gram phenyl glyoxal in 50 cc of water, 5 cc of a chalk suspension and a few drops of toluene. The mixture was incubated for 24 hours at 37°C and mandelic acid extracted in the usual manner. Rotation of solution (10 cc), -0.42° . Glyoxalase was undoubtedly present.

Similar experiments to the above gave negative results when extracts of young potatoes or cultures of *B. bulgaricus* were employed.

STUDIES IN THE BLOOD RELATIONSHIP OF ANIMALS AS DISPLAYED IN THE COMPOSITION OF THE SERUM PROTEINS II

A COMPARISON OF THE SERA OF THE OX, SHEEP, HOG, GOAT,
DOG, CAT AND GUINEA PIG WITH RESPECT TO THEIR
CONTENT OF VARIOUS PROTEINS

By J HOMER WOOLSEY

(From the Rudolph Spreckels Physiological Laboratory of the University of
California)

(Received for publication, April 3, 1913)

In the following investigations I have employed Robertson's refractometric method¹ of determining the concentrations of the various proteins contained in blood sera

The animals employed were in some instances fasted for a definite period preceding the determinations, in the majority of instances normally fed animals were employed and their sera analyzed at an undetermined period after they had last fed. These animals are distinguished below as "normal" while the fasting animals are so designated. The sera were obtained by defibrinating the fresh blood, either by whipping or by shaking up with glass beads, and centrifuging the defibrinated blood. In each case, with the exceptions noted below, the reported analytical results are the average of four closely agreeing determinations made upon the same sample of serum.

A Results obtained with ox serum

The following were the results yielded by four different samples of ox serum obtained from "normal" animals at the time of slaughter in a neighboring meat company's plant

¹ T Brailsford Robertson this *Journal*, 1, p 179, 1912

TABLE I
Ox serum

EXPERIMENT	INSOLUBLE GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
1	0 52 \pm 0 04	1 94 \pm 0 15	5 57 \pm 0 2	7 51 \pm 0 2
2	0 59 \pm 0 04	2 30 \pm 0 15	5 27 \pm 0 2	7 44 \pm 0 2
3	0 62 \pm 0 04	2 34 \pm 0 15	5 18 \pm 0 2	7 52 \pm 0 2
4	0 73 \pm 0 04	2 26 \pm 0 15	5 28 \pm 0 2	7 54 \pm 0 2
Average	0 61 \pm 0 04	2 21 \pm 0 15	5 32 \pm 0 2	7 50 \pm 0 2

The figure following the \pm sign is the possible error in the determination due to a possible error of 1' in reading the angle of total reflection

Expressing each of the above-mentioned proteins in terms of the percentage of the total proteins which they represent, they yield the following figures

"Insoluble" globulin	8 1 { 9 6 6 7 } (\pm 0 4)
Total globulins	29 0 { 31 0 25 0 } (\pm 2 0)
Total albumins	70 0 { 74 0 68 0 } (\pm 2 0)

The first figure opposite each group represents the average percentage, the upper figures immediately following, the highest percentage observed in any individual, the lower figure, the lowest percentage observed in any individual, and the figure in brackets, the plus or minus error in the estimation of these percentages which would be brought about by an error of 1' in reading the angle of total reflection

Previous estimates of the proteins in ox serum are the following

T B Robertson ² Determinations made upon the serum from one animal by the refractometric method

"Insoluble" globulin	8 9 (\pm 0 4)
Total globulins	36 0 (\pm 2 0)
Total albumins	64 0 (\pm 2 0)

O Hammarsten ³ Determinations made upon five animals, globulins precipitated by saturation of the serum with $MgSO_4$, the albumins determined by subtracting the globulins from the total proteins

Total globulins	56 { 64 46
Total albumins	45 { 56 36

² T Brailsford Robertson *loc cit*

³ O Hammarsten *Arch f d ges Physiol*, xvii, p 461, 1878

B Results obtained with sheep serum

The following were the results yielded by four different samples of sheep serum obtained from "normal" animals

TABLE 2
Sheep serum

EXPERIMENT	INSOLUBLE GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
5	0 44 \pm 0 04	1 10 \pm 0 15	6 28 \pm 0 2	7 38 \pm 0 2
6	0 48 \pm 0 04	1 25 \pm 0 15	5 59 \pm 0 2	6 64 \pm 0 2
7	0 46 \pm 0 04	1 07 \pm 0 15	5 47 \pm 0 2	6 55 \pm 0 2
8	0 33 \pm 0 04	1 17 \pm 0 15	4 44 \pm 0 2	5 61 \pm 0 2
Average	0 42 \pm 0 04	1 14 \pm 0 15	5 44 \pm 0 2	6 59 \pm 0 2

Expressing each of the above-mentioned proteins in terms of the percentage of the total proteins which they represent, they yield the following figures

"Insoluble" globulin	6 4 $\left\{ \begin{array}{l} 7 0 \\ 5 9 \end{array} \right\}$ (\pm 0 4)
Total globulins	17 0 $\left\{ \begin{array}{l} 21 0 \\ 14 0 \end{array} \right\}$ (\pm 2 0)
Total albumins	82 0 $\left\{ \begin{array}{l} 85 0 \\ 78 0 \end{array} \right\}$ (\pm 2 0)

Previous estimates of the proteins in sheep serum are the following

J Lewinski ⁴ Globulins precipitated by saturation of the serum with MgSO₄, albumins determined by subtracting the globulins from the total proteins

Total globulins	44
Total albumins	56

C Results obtained with hog serum

The following were the results yielded by four different samples of hog serum obtained from "normal" animals

⁴ J Lewinski *Arch f d ges Physiol*, c, p 611, 1903

TABLE 3
Hog serum

EXPERIMENT	INSOLUBLE GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
9	0 37 \pm 0 04	3 17 \pm 0 15	4 01 \pm 0 2	7 19 \pm 0 2
10	0 48 \pm 0 04	1 98 \pm 0 15	5 21 \pm 0 2	7 19 \pm 0 2
11	0 48 \pm 0 04	3 34 \pm 0 15	4 96 \pm 0 2	8 50 \pm 0 2
12	0 51 \pm 0 04	2 47 \pm 0 15	5 25 \pm 0 2	7 72 \pm 0 2
Average	0 46 \pm 0 04	2 76 \pm 0 15	4 86 \pm 0 2	7 60 \pm 0 2

Expressing each of the above-mentioned proteins in terms of the percentage of the total proteins which they represent, they yield the following figures

"Insoluble" globulin	6 0 { 6 6 5 0 } (\pm 0 4)
Total globulins	36 0 { 44 0 28 0 } (\pm 2 0)
Total albumins	64 0 { 73 0 56 0 } (\pm 2 0)

Previous estimates of the proteins in hog serum are the following

J Lewinski⁵ Globulins precipitated by saturation of the serum with $MgSO_4$, albumins determined by subtracting the globulins from the total proteins

Total globulins	40
Total albumins	60

D Results obtained with goat serum

The following were the results yielded by two different samples of goat serum obtained from "normal" animals

TABLE 4
Goat serum

EXPERIMENT	INSOLUBLE GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
13	0 38 \pm 0 04	1 41 \pm 0 15	5 03 \pm 0 2	6 44 \pm 0 2
14	0 54 \pm 0 04	1 72 \pm 0 15	5 59 \pm 0 2	7 31 \pm 0 2
Average	0 46 \pm 0 04	1 56 \pm 0 15	5 31 \pm 0 2	6 87 \pm 0 2

⁵ J Lewinski *loc cit*

Expressing each of the above-mentioned proteins in terms of the percentage of the total proteins which they represent, they yield the following figures

"Insoluble" globulin	6.5	$\left\{ \begin{array}{l} 7.4 \\ 5.5 \end{array} \right\}$	(± 0.4)
Total globulins	22.0	$\left\{ \begin{array}{l} 23.0 \\ 20.0 \end{array} \right\}$	(± 2.0)
Total albumins	75.0	$\left\{ \begin{array}{l} 76.0 \\ 73.0 \end{array} \right\}$	(± 2.0)

Previous estimates of the proteins in goat serum are the following

C Quinan * Determinations made upon one animal at intervals of one month in four series of analyses, "insoluble" globulin precipitated by CO_2 , albumins determined by N determinations upon filtrate freed of globulins by MgSO_4 , globulins determined by subtracting the albumins from the total proteins

"Insoluble" globulin	9.4
Total globulins	46.0
Total albumins	54.0

E Results obtained with dog serum

The following were the results yielded by five different samples of dog serum obtained from animals fasted for forty-eight hours. In each case the reported analytical results are the average of three closely agreeing determinations upon the same sample of serum

TABLE 5
Dog serum

EXPERIMENT	INSOLUBLE GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
15	0.38 ± 0.04	0.81 ± 0.15	5.66 ± 0.2	6.47 ± 0.2
16	0.57 ± 0.04	1.25 ± 0.15	6.24 ± 0.2	7.50 ± 0.2
17	0.59 ± 0.04	1.79 ± 0.15	4.98 ± 0.2	6.72 ± 0.2
18	0.67 ± 0.04	1.39 ± 0.15	6.69 ± 0.2	8.08 ± 0.2
19	0.55 ± 0.04	1.31 ± 0.15	5.24 ± 0.2	6.57 ± 0.2
Average	0.55 ± 0.04	1.31 ± 0.15	5.76 ± 0.2	7.07 ± 0.2

* C Quinan *Univ of Calif Publ Path*, 1, p 1, 1903

Expressing each of the above-mentioned proteins in terms of the percentage of the total proteins which they represent, the following figures are obtained

"Insoluble" globulin	7.7	$\left\{ \begin{array}{l} 8.8 \\ 5.9 \end{array} \right\}$	(± 0.4)
Total globulins	18.0	$\left\{ \begin{array}{l} 27.0 \\ 12.0 \end{array} \right\}$	(± 2.0)
Total albumins	81.0	$\left\{ \begin{array}{l} 87.0 \\ 74.0 \end{array} \right\}$	(± 2.0)

Previous estimates of the proteins in dog serum are the following

J Lewinski ⁷ Globulins precipitated by saturation of the serum with $MgSO_4$, albumins determined by subtracting the globulins from the total proteins

Total globulins	42
Total albumins	58

G Salvioni ⁸ Globulins precipitated by saturation of the serum with $MgSO_4$, albumins by subtracting the globulins from the total proteins

Total globulins	$37 \left\{ \begin{array}{l} 49 \\ 23 \end{array} \right\}$
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F Results obtained with cat serum

The following were the results yielded by four different samples of cat serum obtained from animals fasted for forty-eight hours. Two determinations were made upon each sample of serum

TABLE 6
Cat serum

EXPERIMENT	INSOLUBLE GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
20	0.64 ± 0.04	4.09 ± 0.15	5.0 ± 0.2	9.09 ± 0.2
21	0.48 ± 0.04	2.09 ± 0.15	6.02 ± 0.2	8.11 ± 0.2
22	0.55 ± 0.04	2.03 ± 0.15	6.32 ± 0.2	8.35 ± 0.2
23	0.52 ± 0.04	2.05 ± 0.15	6.10 ± 0.2	8.15 ± 0.2
Average	0.55 ± 0.04	2.56 ± 0.15	5.86 ± 0.2	8.42 ± 0.2

⁷ J Lewinski *loc cit*

⁸ G Salvioni *Arch f (Anat u) Physiol*, 1881, p 269

Expressing each of the above-mentioned proteins in terms of the percentage of the total proteins which they represent, the following figures are obtained

"Insoluble" globulin	6 5	$\left\{ \begin{array}{l} 7\ 1 \\ 5\ 9 \end{array} \right\}$	($\pm 0\ 4$)
Total globulins	30 0	$\left\{ \begin{array}{l} 45\ 0 \\ 24\ 0 \end{array} \right\}$	($\pm 2\ 0$)
Total albumins	69 0	$\left\{ \begin{array}{l} 76\ 0 \\ 55\ 0 \end{array} \right\}$	($\pm 2\ 0$)

G Results obtained with guinea pig serum

The following were the results yielded by one sample of guinea pig serum obtained from ten animals fasted for twenty-four hours. Two determinations were made upon the sample of serum

TABLE 7
Guinea pig serum

EXPERIMENT	INSOLUBLE GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
24	0 25 \pm 0 04	0 93 \pm 0 15	5 01 \pm 0 2	5 94 \pm 0 2

Expressing each of the above-mentioned proteins in terms of the percentage of the total proteins which they represent, the following figures are obtained

"Insoluble" globulin	4 2 ($\pm 0\ 4$)
Total globulins	16 0 ($\pm 2\ 0$)
Total albumins	84 0 ($\pm 2\ 0$)

SUMMARY

The following table summarizes the average results obtained

	PERCENTAGE OF THE TOTAL PROTEINS IN THE SERA OF						
	Ox	Sheep	Hog	Goat	Dog	Cat	Guinea Pig
"Insoluble" globulin	8 1	6 4	6 0	6 5	7 7	6 5	4 2
Total globulin	29 0	17 0	36 0	22 0	18 0	30 0	16 0
Total albumin	70 0	82 0	64 0	75 0	81 0	69 0	84 0

STUDIES ON THE THEORY OF DIABETES

I SARCOLACTIC ACID IN DIABETIC MUSCLE

By R T WOODYATT

(From the Otho S A Sprague Memorial Institute, Laboratory of Clinical Research, Rush Medical College, Chicago)

(Received for publication, April 7, 1913)

The primary chemical disability in diabetes mellitus and pancreas diabetes has been held by different writers to affect each of the things which it is known can be done with sugar by a normal body, *e g*, to burn it, to produce it in moderation, to store it as fat, or to "fix" it as glycogen, etc. As far back as 1871 Schultzen suggested that the primary error was an inability to open up the glucose molecule and so render it fit for oxidation.

For a number of years we have found it serviceable to regard dissociation of sugar as prerequisite for its chemical reactivity in general, *i e*, for its oxidation, reduction, polymerization, combination, or functioning in equilibria—in the same sense that this has been made the basis for a great array of organic chemical reactions *in vitro* by J U Nef. The active principle in the so-called internal secretion of the pancreas is a substance whose effect, like that of alkali *in vitro*, is to increase the dissociation of certain classes of substances including the sugars. Once dissociated, the fate of the residues thus set free depends upon the reaction conditions encountered by these residues in the various cells and fluids of the body.

Sarcolactic acid has a peculiar interest in this connection because it may be formed in the body under certain circumstances (*i e*, under conditions tending toward asphyxia—general or local), and because in so far as it comes from sugar it represents purely and simply a product of dissociation and intramolecular rearrangement. This interpretation of lactic acid as a dissociation product of glucose has been made by a number of writers. The question arises. Is the power of the body to form lactic acid out of sugar lessened in diabetes?

It is a fact that lactic acid is seldom found in detectable quantities in the ether extracts so frequently obtained in the routine determination of β -hydroxybutyric acid in the urine of severe diabetes. Von Noorden¹ states that he has three times sought it in vain. We have also failed to prepare an insoluble zinc salt from this residue in a number of attempts. The reason for this is clear when one recalls the work of Mandel and Lusk² who demonstrated the complete conversion of administered lactic acid into sugar in fully phlorhizinized dogs and its failure to occur even in the urine of dogs which were poisoned with phosphorus when these dogs were fully phlorhizinized. Obviously one can draw no conclusion from the urinary findings as to the diabetic's ability to form lactic acid. There are other ways, however, of attacking the problem.

Fletcher and Hopkins³ have held that the lactic acid which is formed in fresh muscles, and which has been regarded by many as intimately connected with rigor mortis and death, is a product solely of the survival period. Under proper circumstances a given weight of muscle will produce a certain maximum quantity of lactic acid, after which no injury or stimulation will influence it to form any more. (Lactic acid can also be formed according to Kondo⁴ by the action of fresh muscle juice and the quantity formed can be varied by altering the reaction of the juice. The addition of alkali according to him prolongs and increases, the addition of acid shortens and decreases the lactic acid formation. He believes that the degree of acidity may set the limit for survival lactic acid formation. But this does not imply that with a given surviving muscle the quantity of lactic acid produced is not a function of the mother substance from which it arises.) Now since the lactic acid formed in an isolated muscle is not subjected to the drain of sugar which occurs during life when glycosuria is in progress and which tends to "draw" into glucose the lactic acid, alanine, glycerol, and other substances which are normally in equilibrium with glucose, we decided to produce diabetes in animals, study their metabolism during life, and then ascertain the power of the isolated muscle to form lactic acid during the survival period.

¹ *Handbuch der Pathologie des Stoffwechsels*, 1907, II, p. 95

² Mandel and Lusk. *Amer. Journ. of Physiol.*, xvi, p. 129, 1906

³ *Journ. of Physiol.*, xxxv, p. 247, 1907

⁴ *Biochem. Zeitschr.*, xlv, p. 63, 1912

Methods

The method employed for securing a maximum survival yield of lactic acid was devised in accordance with the principles established in the papers of Fletcher and Hopkins⁵ and of Fletcher⁶. According to them mammalian muscles are tardy in reaching their final acidity unless hurried by methods which provide a suitable temperature and stimulus without premature injury. Absence of oxygen, the proper time and temperature, certain chemicals, *e g*, chloroform, insure completeness of the process. The animals used in these experiments were deeply anaesthetized with chloroform. The abdomen was then opened and portions of liver rapidly removed for glycogen determinations, which were carried out in accordance with the directions of Pflüger⁷. The great abdominal vessels were then slashed and the animal suspended by the hind limbs to insure as complete exsanguination of the latter parts as possible. Muscle was then rapidly dissected from the fore-quarters for glycogen determination. This done, the carcass was immersed in water at 45°C for an hour, then removed from the water and kept in a covered boiler for six hours more at 30–35°C. Rigor mortis always developed fully during the first hour. At the end of the six hours the skin was dissected from the hind limbs, after which the muscles were dissected out cleanly, care being taken to use the same groups on each side. The muscles were then run through a mincer, after which the minced muscle was thoroughly mixed. The mincing and mixing were done four times in all, after which the hash was weighed out into 50-gram portions, each of which was covered with 500 cc of 95 per cent alcohol.

The subsequent steps in the alcohol extraction, the removal of fat and lipoids by means of blood charcoal, the ether extraction out of the acidified aqueous solution, the conversion of the lactic acid into the zinc salt, its weighing in the anhydrous form, as well as the determination of its purity by means of ZnO determinations, were all done essentially as described by Fletcher and Hopkins. We introduced, however, certain mechanical changes.

Thus, in the original alcohol extraction we simply stirred the hashed muscle with a rod until all clumps were thoroughly separated and after

⁵ *Journ of Physiol*, xxxv, p 247, 1907

⁶ *Ibid*, xliii, p 286, 1911

⁷ Abderhalden *Handbuch der Biochem Arbeitsmethoden*, 1910, ii, p 1071

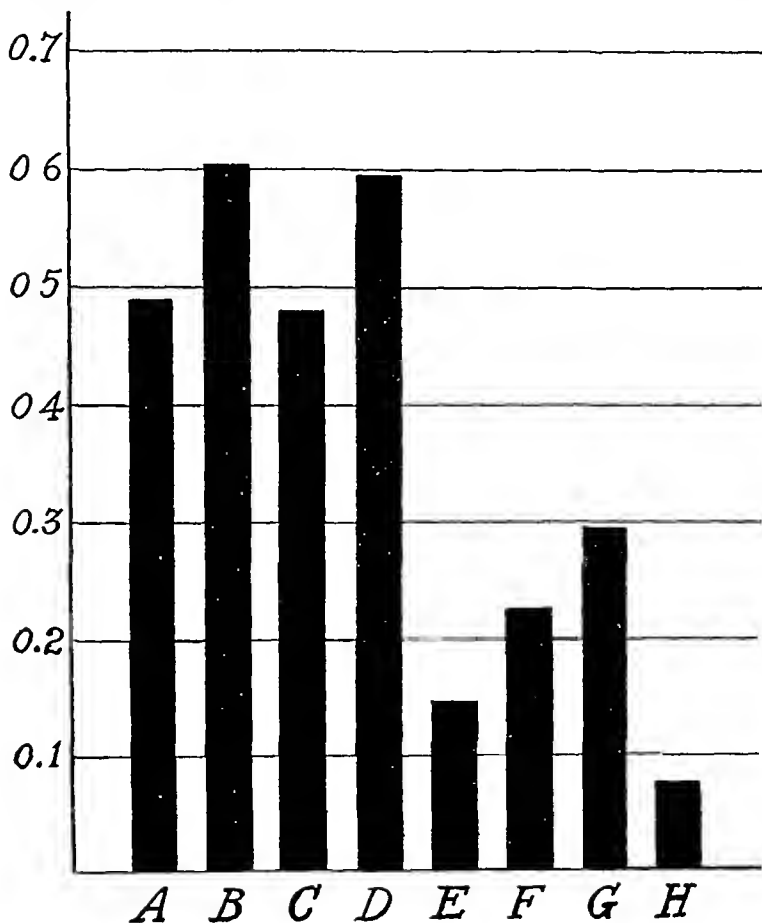
twenty-four hours' standing decanted the alcohol on to a small, folded filter (Fletcher and Hopkins rubbed up the muscle and alcohol in a mortar and squeezed out the muscle mass in muslin after decanting, making, in all, four extractions) After five extractions, each with 500 cc of 95 per cent alcohol, the muscle residue was allowed to dry in a desiccator, then pulverized in a mortar and finally extracted twice more as before In clearing with blood charcoal to remove lipoids, etc, we made the aqueous solution of the alcoholic residue to exactly 150 cc, then added 7-10 grams of blood charcoal and carried out the heating under an efficient reflux condenser After cooling, the liquid was filtered, and an aliquot part of the whole was used for analysis, generally 120 cc or 80 per cent This avoided the process of washing and reboiling the charcoal, employed by Fletcher The filtrate was then evaporated to dryness, made up with H_2O to exactly 20 cc in a bulbed graduate ("phenol tube") and then to 25 cc with a saturated solution of phosphoric acid This solution was covered with 100 cc of ether and shaken In separating the ether layer we employed a special siphon instead of a pipette and carried out the extraction as stated in a "phenol tube," the bulb of which permitted the use of a large volume of ether for the 25 cc of solution, although the graduated stem of the tube was narrow and permitted of a very sharp separation of layers We extracted the aqueous residue ten times with exactly 100 cc of ether each time, and proceeded in the same way in each experiment The ether subsequent to decantation was handled as in Magnus-Levy's method for β -hydroxybutyric acid determination in the urine, to ensure freedom from emulsified solution containing inorganic acid The final zinc salt was always white and pure and when analyzed gave uniformly satisfactory ZnO figures, so that in some experiments we omitted this final ignition Analyses were made in pairs

Phlorhizinization of dogs was accomplished by administering to animals of 7-9 kgms body weight, every 6 hours subcutaneously, 2 grams of phlorhizin dissolved in 30 cc of 1.2 per cent solution of sodium carbonate During the first hours of the regimen the dogs were given cold baths and permitted to shiver afterwards, according to Lusk's method The urine was obtained by catheter and the bladder irrigated with warm water at the end of each 6-hour period Nitrogen determinations in the urine were made by the Kjeldahl method, dextrose by polariscope, and by the titration method of Bang and Bohmannsson⁸

Results

The relative quantities of lactic acid obtained per 100 grams muscle, and expressed in weights of anhydrous zinc lactate may be seen by reference to the chart The first four lines, A, B, C,

and *D*, represent the quantity of zinc lactate obtained from the lactic acid in 100 grams of muscle from healthy dogs, in such states of nutrition as they were when brought to the laboratory. The last line *H* is the figure obtained in a case of severe human diabetes. The other three lines, *E*, *F*, *G*, correspond to the figures obtained from muscles of phlorhizinized dogs, the lowest, *E*, being that from a fully phlorhizinized and glycogen-free animal in which the D/N ratio was 3.73/1, the next lowest, *F*, a dog in which the ratio was 2.82/1 and in which a trace of glycogen was found, the highest



of the three, *G*, a dog in which the ratio was 2.90 : 1, but to which glucose was given intravenously just before death in order to create an hyperglycaemia, and which contained in the muscles 0.22 per cent glycogen

Discussion of results

It is apparent from these figures that the quantity of lactic acid formed in muscles from healthy dogs varies, presumably with the different states of nutrition. It is also clear that the muscle from the human diabetic formed distinctly less lactic acid than the lowest normal muscle. But it is very possible that the survival lactic acid comes from three sources (1) glycogen sugar, (2) sugar derived from protein or fat, (3) directly from fatty or amino-acids without intermediate sugar formation. In the case of the muscle of the human diabetic, the fraction (1) from glycogen might naturally be expected to be absent or very low because in such diabetics the muscles contain little or no glycogen. Therefore the muscle of the human diabetic had presumably less material to work upon than the normal and the values obtained are not directly comparable to those found for muscles of well nourished animals. In Experiment *E* the factor (1) was removed by means of phlorhizin, thus producing a state of nutrition parallel to that found in human diabetes but nevertheless without injury to the pancreas. Incidentally an hypoglycaemia was caused, i.e., the value of (2) was somewhat lowered. The lactic acid formed by the muscle of this dog is therefore directly comparable to that found in the human, except for the difference in species and perhaps in the lessened value of (2).

In Experiment *F* the attempt was made to replace (2) by creating hyperglycaemia just before death, but in such experiments there is a rise both in the amount of glycogen and of lactic acid found. This experiment appears to demonstrate directly the power of the muscle to form lactic acid and glycogen from glucose, and affords possibly the best comparison of the series with the muscle of the human diabetic. Now if we compare the lactic acid formed in human diabetes with that formed in the phlorhizinized dogs (in which pancreatic function was normal) it appears that with the same material at hand out of which to form lactic acid or even with a little less at hand, the phlorhizinized

dog muscle made somewhat more lactic acid than that of the human diabetic

In all cases in which diabetic muscles were analyzed there was some lactic acid formation. This would have to be ascribed to protein (and perhaps in part to fat)—either directly or through sugar as an intermediate. Owing to this fact—that lactic acid formed in surviving muscles comes partly from sources other than sugar—lactic acid cannot be made a direct measure of the dissociation of sugar in these experiments as was originally hoped.

As an incident it is interesting to note that a dog, in which a D N ratio of 3.73:1 was produced, proved to be in reality glycogen-free by post-mortem analysis, a confirmation of the conclusion reached by Lusk.⁹ The animal in which the ratio was 2.83 showed a trace of glycogen in the muscles. And with a ratio of 2.97 and the intravenous administration of glucose the glycogen in the muscle was 0.22 per cent.

EXPERIMENTS

I

Experiments with healthy dogs in different states of nutrition handled in accordance with methods described on p. 443

EX- PERI- MENT	TOTAL $\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2$ (ANHYDROUS) PER 100 GMS. MUSCLE	AMOUNT $\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2$ USED FOR INCINERATION	ZNO		
			Weight obtained	Per cent	Theory
A	0.491	0.321	0.109	34.24	33.43
B	0.605				
C	0.481				
D	0.597	0.235	0.085	36.17	33.43

⁹ *Elements of the Science of Nutrition*, 1909, second edition

II

Experiments with phlorhizinized dogs—phlorhizinized and handled as described under Methods, p 444

EXPERIMENT E The urinary findings for successive 6-hour periods gave the following figures (Dextrose, Bang-Bohmannsson, N, Kjeldahl)

PERIOD	DEXTROSE	N	D N	AVERAGE D N FOR LAST 18 HOURS
	<i>per cent</i>	<i>per cent</i>		
I	2 50	0 35	7 14	} 3 73 1
II	4 04	0 57	7 09	
III	2 90	0 58	5 00	
IV	3 50	0 97	3 60	
V	3 86	0 96	4 00	
VI	3 59	1 00	3 59	

Glycogen determinations carried out on 50 grams each of muscle and liver (Pflüger) The final residue obtained after filtration of the alcohol was dissolved in 20 cc of hot water and cleared with HCl as per Pflüger's directions This fluid showed no dextro-rotation and, after boiling for one half hour with 10 per cent HCl and neutralization, no reducing power in Haines' solution The iodine reaction was also negative

Total anhydrous $Zn(C_2H_3O_2)_2$ obtained per 100 grams muscle, 0 1467 gram Used for ZnO determination, 0 0514 gram ZnO found, 0 0172—corresponding to 33 46 per cent, theory, 33 43 per cent A blood-sugar determination (Bang, Lyttkens and Sandgren, *Zeitschr f physiol Chem*, lxx, p 497) showed 0 073 per cent

EXPERIMENT F *Phlorhizinized dog* The same procedure as in E, except that no effort was made to collect the whole urine for each period nor to maintain even dilution of the urine

PERIOD	DEXTROSE	N	D N	AVERAGE D N FOR LAST 18 HOURS
	<i>per cent</i>	<i>per cent</i>		
I				} 2 82 1
II	2 55	0 46	5 59	
III	1 93	0 67	2 88	
IV	1 82	0 69	2 64	
V	1 80	0 61	2 94	

Glycogen determination in muscle, trace, in liver, trace (?)

Total anhydrous $Zn(C_2H_3O_2)_2$ per 100 grams muscle, 0 227 gram Used for ZnO determination, 0 0782 gram ZnO found, 0 0263, corresponding to 33 65 per cent, theory, 33 43 per cent

EXPERIMENT G *Phlorhizinized dog with intravenous glucosc injection*

PERIOD	DEXTROSE	N	D N	AVERAGE D N FOR LAST 18 HOURS
	<i>per cent</i>	<i>per cent</i>		
II	4 17	0 85	4 85	} 2 97
III	4 17	1 57	2 66	
IV	2 50	0 74	3 37	
V	2 40	0 83	2 90	

At the close of the last period analyses of the urine were made as usual, the time consumed being two hours. The animal was then chloroformed and 100 cc. of a 12.9 per cent glucose solution introduced into the femoral vein. The ureters were then clamped to stop excretion. Ten minutes later the usual procedure was carried out.

Glycogen determination in liver, a trace (the residue from 50 grams liver, made up in the final step to 50 cc. with water, read in a 20 cm. tube + 0.06°). Glycogen in the muscle, 0.219 per cent (final residue from 50 grams of muscle made up to 20 cc. read in 20 cm. tube + 1.09°).

III

Human diabetes (so-called "bronze diabetes")

EXPERIMENT H *Clinical history* Dr. G. P., age 42, case No. 61501, Presbyterian Hospital, Chicago. Service of Dr. Frank Billings. Patient entered the hospital June 15, 1911, and died in true dyspnoeic coma ten weeks later. He probably had a transient glycosuria four years prior to admission. Four months before admission a controllable glycosuria occurred. One month before admission it became intractable. On admission the urine contained much sugar, acetone, acetoacetic and β -hydroxy-butyric acids with a high NH_4 figure. He complained of weakness, loss of weight and strength, thirst and polyuria. Just before this time because of an old luetic affair he had taken a course of baths and mercurialunctions after which he was troubled greatly with pain in the distribution of the sacral nerves.

Physical examination on admission Patient is 5 feet 10 inches tall and weighs only 102 pounds. The skin is a little dusky—suggesting pigmentation. The liver presents a hard, sharp and smooth edge 2.5 inches below the costal arch in the mamillary line. The tip of the spleen is palpable. The arteries are somewhat sclerosed. Otherwise the findings have no bearing in this place. Wassermann, negative.

Subsequent history He was frequently in trouble from the start with headache, slight nausea, drowsiness, backache and a rising acidosis if he attempted to move far from his bed or room.

At one time he was given an analyzed diet—containing protein, 85 grams, wheat starch, 75 grams (or 100 grams as noted), fat, 170 grams, aggregating

(approximately) 2170 Cal In addition to this he received sodium bicarbonate, 20 grams, and claret, 400 cc *per diem*

The findings follow

DAY	DIET	WEIGHT <i>pounds</i>	AMOUNT	SPECIFIC GRAVITY	DEXTROSE		NH ₄	N	Q	Z
					Per cent	Total				
23	As stated above	105	4330	1025	2 43	105	4 4	13 4	852	2 2
24	Same	105	4425	1027	2 53	111	3 5	18 6	771	9
25	Same except starch made to 100 gm	105	4725	1025	2 82	133	3 6	19 0	781	7
27	Same as on 25	105	4700	1025	2 93	133	7 5	18 9	771	7
28	Interrupted	105	5100	1026			8 1			

$$Q = \frac{\text{Urinary glucose} \times 100}{(\text{Urinary N} \times 3.65) + \text{carbohydrate ingested}}$$

$$\frac{D}{N} = \frac{\text{Urinary glucose} - \text{carbohydrate ingested}}{\text{Urinary N}}$$

On the 26th he ran a slight temperature ascribed to exacerbation of an old prostatitis and on the 27th the NH₄ figure was extreme. A fast day was therefore interpolated with a simultaneous increase of the wine and alkali and addition of opium. There was temporary improvement but thereafter his condition was always critical and demanded frequent alterations of the diet. Analysis of the food, etc., was consequently discontinued. The usual clinical resources were employed. He could be held in N equilibrium but not without the ingestion of much fat and the appearance of threatening symptoms. From August 12 to 20, his diet averaged approximately 100 grams of carbohydrate, 125 grams protein, 200 grams fat, supplemented with wine, 0.5 to 1 liter, and sodium bicarbonate, 15 to 20 grams. The routine examination of the urine for this period ran as follows:

DAY	AMOUNT URINE	SPECIFIC GRAVITY	GLUCOSE	NH ₄
12	3950	1019	71	2 8
13	3000	1026	108	1 9
14	3800	1026	159	1 9
15	4325	1029	164	2 4
16	3500	1026	108	2 3
17	3800	1030	144	2 5
18	3425	1030	145	2 5
19	4300	1032	168	3 0
20	4450	1030	165	3 9

Assuming the daily average carbohydrate ingestion to have been 100 grams and the average urinary N to have been 20 grams (which cannot be far from what occurred) the Q for the last nine days would have been 77 as in the previous period. *The case represents therefore a "severe" but "incomplete" diabetes*

Autopsy Performed four hours after death. Body still warm. The liver and pancreas show the typical sclerosis and brick-red parenchyma of "bronze diabetes." Psoas muscle taken for analysis was kept four hours in a warm place, then handled as usual.

Total anhydrous Zn(C₃H₅O₂) per 100 grams muscle, 0.077 gram

SUMMARY

Muscles of glycogen-free animals form some sarcolactic acid (about 30 per cent of the normal). This lactic acid cannot come from glycogen but must arise from preformed sugar, or directly from certain amino- or fatty-acids. The muscle of a case of severe human diabetes formed even less lactic acid than that of fully phlorhizinized dogs. This suggests an impaired power to dissociate glucose on the part of the diabetic muscle since such muscles are bathed with an abnormally high quantity of sugar which—if available—should yield more lactic acid than is found in the muscle of phlorhizin diabetes.

With D/N ratios of approximately 3.65/1, post-mortem analyses of dog muscles and livers show no glycogen. With ratios of 2.8 or 3.0/1 this is not necessarily the case, and one cannot assume that with a constant D/N ratio of 2.8/1 an animal is free of glycogen.

ON THE ABSORPTION OF NITROGENOUS PRODUCTS— A REPLY TO ABDERHALDEN AND LAMPÉ

BY OTTO FOLIN AND W. DENIS

(From the Biochemical Laboratory of Harvard Medical School, Boston)

(Received for publication, April 9, 1913)

Some time ago Abderhalden and Lampé published a paper¹ on the absorption of amino-acids, peptones, etc., which is largely devoted to a review and criticism of our experimental results and conclusions on the same subject.²

In this paper Abderhalden presents his views as to the nature of the problem, the relative merits of the theories in vogue with regard to absorption, the difficulties involved, etc., and reviews the bearing on these questions of his own researches including some new experiments which so far as can be judged from the rather sketchy description and the protocols, represent qualitative control experiments of our absorption determinations. The results recorded we interpret as a very acceptable confirmation of our facts and interpretations. So far as our work is concerned, however, Abderhalden admits that our analytical methods are new, but considers the results which we obtained neither new nor particularly illuminating.

The theory of amino-acid absorption accompanied by immediate deamination is according to Abderhalden superfluous to discuss because it lacks experimental support. (Our experiments have shown the immediate deamination hypothesis to be untenable.) The theory of amino-acid absorption and subsequent distribution to the different tissues as presented in our papers Abderhalden considers not yet proven, in other words, this is still a theory and not an established fact. Even as a theory he is inclined to be more critical of it than of his own "Arbeitshypothese"—the immediate regeneration of blood proteins out of the amino-acids. The

¹ *Zeitschr. f. physiol. Chem.*, lxxxi, p. 473, 1912.

² This *Journal*, vi, p. 87, 1912, vii, p. 141, 1912.

regeneration doctrine, though it too is only a hypothesis and without a particle of positive experimental evidence in its favor, he nevertheless thinks should be supported until *facts* have made it superfluous (pp 495-6) Just why this one hypothesis should receive the benefit of all remaining doubts he does not make clear, but since he still takes that position others, like ourselves, feel justified in referring to him as the chief exponent and defender of it The fact that he in "1912" mentioned its weakness from an experimental standpoint (Folin made clear its weakness in 1905)¹ does not alter the fact that it is still his working hypothesis, though perhaps in a modified form If we understand him rightly his working hypothesis now is that those amino-acids which cannot be built up into blood protein may get into the blood as amino-acids

While Abderhalden thus remains avowedly a supporter of the immediate regeneration hypothesis as a feature of absorption it is clear that his experimental results described in his paper with Lampé confirm our findings so far as such qualitative tests can confirm them By qualitative tests using 10 cc of blood he is now able to demonstrate the presence of amino-acids in the blood at all times and an unmistakable increase in the reaction as a result of a few minutes' absorption of any ordinary amino-acid In his earlier work Abderhalden was unable to prove the presence of amino-acids in blood even when he worked with as much as 50 liters at a time

Abderhalden's severely critical attitude toward our results and interpretations appears to be a consequence of his bias in another direction To those who for years have recognized that the protein regeneration doctrine had only an old teleological reason, based on false premises, to support it, and who consequently regarded the immediate deamination theory as more probable and reasonable, our facts and interpretations will appear in a different light

As for ourselves, we hold that our experimental results transform into a demonstrable fact the theory of amino-acid absorption unaccompanied by immediate deamination or protein regeneration We recognize that our facts need verification, and any one who will learn to use our quantitative methods can easily repeat our work

¹ *Amer Journ of Physiol*, xii, p 117, 1905

Abderhalden constantly overlooks the *quantitative* aspect of our results as when he says that these have no more significance than has the presence of traces of glycerin in the blood in connection with the problem of fat absorption, or when he says that we must apply his and Lampé's qualitative "indirekten Bestimmungen" (p 491) in order to obtain a correct interpretation of our results

By the help of Abderhalden's favorite reagent, triketo hydrin hydrate, or nin hydrin as he now calls it, we have no doubt that additional interesting confirmatory tests may be obtained, and we hope that he will continue to use it, and that he will also apply it to the study of the absorption of amino-acids from the stomach.⁴ Since there is not any reason direct or indirect, theoretical or experimental, for assuming that our quantitative results on the absorption and distribution of amino-acids are less valid than our corresponding results with urea, creatine and creatinine, we regard the qualitative tests demanded by Abderhalden as interesting and valuable to be sure, but nevertheless only as merely confirmatory. In the case of one amino-acid, tyrosine, we did substitute a qualitative test and by its help were able to trace its passage into the blood and muscles, a fact which Abderhalden seems to have overlooked.

In conclusion we think that Abderhalden interpreted somewhat unfairly the reservation of the field opened by our technique. The reservation manifestly referred only to our methods of studying the problem, *i e*, by the help of our new analytical methods. And it was evidently thus understood by Van Slyke and Meyer,⁵ the first to bring out facts which verify our findings. Moreover in the paper describing the method, only three months later, a reprint of which was in Abderhalden's possession, we revoked the reservation in the most unmistakable manner. "We hereby expressly revoke our earlier reservation of the field of research referred to in those papers by means of these methods."

⁴ Comment on Abderhalden's criticisms on the demonstration of absorption from the stomach by Folin and Lyman is superfluous since in a reply to London (this *Journal*, xiii, p 389, 1912) that matter was adequately covered.

⁵ This *Journal*, xii, p 399, 1912.

ON THE TYROSINE CONTENT OF PROTEINS— A REPLY TO ABDERHALDEN AND FUCHS

By OTTO FOLIN AND W DENIS

(From the Biochemical Laboratory of Harvard Medical School, Boston)

(Received for publication, April 9, 1913)

In a recent paper by Abderhalden and Fuchs,¹ entitled "Über den Gehalt der Proteine an l-Tyrosin und die Genauigkeit der Bestimmung dieser Aminosäure" the closing sentence reads as follows

"Die kolorimetrische Methode von Folin und Denis vermag die Bestimmung des Tyrosins durch Krystallisation nicht zu ersetzen, weil sie auch andere Aminosäuren nachweist und infolgedessen zu hohe Werte liefert "

The statement contained in the above sentence is given as though it embodied a conclusion derived from the experimental work described in the paper whereas in point of fact it represents only an opinion. The only observation recorded by Abderhalden and Fuchs in support of that opinion is that tryptophane and a certain obscure substance ("oxytryptophan") which, so far as we know no one but Abderhalden has yet observed, also give a slowly developing blue color with our tyrosine reagent.

We omitted to apply our color reaction to tryptophane partly because we had none on hand and partly because we assumed (perhaps incorrectly) that very little tryptophane would survive the treatment with boiling mineral acids which we employed for splitting off the tyrosine. Further, it is to be noted that the excess of tyrosine indicated by our method is no greater in the case of casein with 1.5 per cent tryptophane than in the case of zein which is supposed to have none. For the former we obtained 6.5 per cent as against 4.5 per cent obtained by E. Fischer, for the latter our figure is 5.5 per cent as against Osborne's 3.6 per cent.

¹ *Zeitschr f physiol Chem*, lxxiii, p 468, 1913

On the strength of Abderhalden's observation we nevertheless concede for the time being at least that our tyrosine figures may be somewhat high though there is clearly no reason to believe that an adequate explanation of the difference between our figures and those recorded in the literature are to be explained by the presence of tryptophane. Our comparisons were made with the tyrosine figures already in the literature including several of Abderhalden's. If Abderhalden and Fuchs have now (for the first time) succeeded in working out a method for a quantitative isolation of pure tyrosine, that is a different matter, and when Abderhalden on the basis of his new method has published revised figures for the tyrosine content of at least some proteins, we shall perhaps know whether our figures represent tyrosine or tyrosine plus something else. Until he has done so, however, we would rather insist that our tyrosine figures are more nearly correct than those heretofore recorded in the literature.

THE INFLUENCE OF BASES UPON THE RATE OF OXIDATIONS IN FERTILIZED EGGS

BY JACQUES LOEB AND HARDOLPH WASTENEYS

(From the Rockefeller Institute for Medical Research, New York)

(Received for publication, April 18, 1913)

1 In a previous paper we have shown that the weak bases cause a more rapid and greater increase in the rate of oxidations in the *unfertilized* egg than the strong bases¹ This result differed in principle from the results which Warburg obtained while working on *fertilized* eggs² He found that NH_4OH does not increase the rate of oxidations in *fertilized* eggs of *Strongylocentrotus lividus* at Naples, while NaOH does, and since NH_4OH diffuses into the egg while NaOH does not, he concludes from this that the effect of OH ions on oxidations is neither determined by their diffusion into the egg nor through their reaction with the plasma membrane, but merely through their presence in the solution surrounding the cell (p 316) This conclusion does not harmonize with the results which we obtained with the unfertilized egg, in which NH_4OH influenced the oxidations more strongly than the NaOH We therefore made some experiments on *fertilized* eggs in order to clear up if possible this apparent contradiction This seemed sufficiently important since it affects not only the theory of fertilization but also the problem of the localization of oxidations in the cell

Warburg only published the results of three determinations of the rate of oxidations at three different concentrations of NaOH in a mixture of $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ in that proportion in which these salts occur in the sea water His results were as follows

TABLE I

Rate of oxidations in fertilized eggs of *Strongylocentrotus lividus* at Naples

C_{NaOH}	COEFFICIENT OF OXIDATIONS	
10^{-3}N	1 4	No segmentation
10^{-6}N	3 9	Normal segmentation
10^{-3}N	8 1	No segmentation

¹ Loeb and Wasteneys this *Journal*, xiv, p 355, 1913

² O Warburg *Zeitschr f physiol Chem*, lxxvi, p 305, 1910

It seemed of importance to us to ascertain whether indeed the rate of oxidations in fertilized eggs increased steadily with the increase in the concentration of NaOH. For this reason experiments were carried on with the *fertilized* eggs of *Strongylocentrotus purpuratus* in California. Of freshly fertilized eggs of one female a homogeneous suspension was made and this was divided into five equal lots which were then distributed into five different solutions of NaCl + KCl + CaCl₂ (in the proportion in which these salts exist in the sea water) and various quantities of $\frac{N}{10}$ NaOH were added.

TABLE II

NATURE OF THE SOLUTION	OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
	mgm	
Neutral NaCl + KCl + CaCl ₂	0 44	1 00
50 cc NaCl + KCl + CaCl ₂ + 0 3 cc $\frac{N}{10}$ NaOH	0 48	1 03
50 cc NaCl + KCl + CaCl ₂ + 0 4 cc $\frac{N}{10}$ NaOH	0 48	1 03
50 cc NaCl + KCl + CaCl ₂ + 0 5 cc $\frac{N}{10}$ NaOH	0 50	1 13
50 cc NaCl + KCl + CaCl ₂ + 0 6 cc $\frac{N}{10}$ NaOH	0 52	1 18
Neutral NaCl + KCl + CaCl ₂	0 47	1 00
50 cc NaCl + KCl + CaCl ₂ + 0 7 cc $\frac{N}{10}$ NaOH	0 55	1 17
50 cc NaCl + KCl + CaCl ₂ + 0 8 cc $\frac{N}{10}$ NaOH	0 71(?)	1 51(?)
50 cc NaCl + KCl + CaCl ₂ + 0 9 cc $\frac{N}{10}$ NaOH	0 68	1 44
50 cc NaCl + KCl + CaCl ₂ + 1 0 cc $\frac{N}{10}$ NaOH	0 73	1 55
Neutral NaCl + KCl + CaCl (two determina- tions)	0 41	1 81 1 93 2 12 (eggs injured)
	0 43	
50 cc NaCl + KCl + CaCl ₂ + 1 1 cc $\frac{N}{10}$ NaOH	0 76	
50 cc NaCl + KCl + CaCl ₂ + 1 2 cc $\frac{N}{10}$ NaOH	0 81	
50 cc NaCl + KCl + CaCl ₂ + 1 3 cc $\frac{N}{10}$ NaOH	0 89	
50 cc NaCl + KCl + CaCl ₂ + 1 4 cc $\frac{N}{10}$ NaOH	0 79	

These results seem to indicate one point clearly, namely, that the addition of 0 5 cc of $\frac{N}{10}$ NaOH or less to 50 cc of salt solution has practically no effect upon the rate of oxidations. This experiment was repeated (table III).

The experiment confirms the result of the preceding series. The addition of 0 4 cc or less of $\frac{N}{10}$ NaOH to 50 cc of salt solution has

no effect upon the rate of oxidations in the fertilized egg, and the addition of 0.8 cc of $\frac{N}{10}$ NaOH has only a very slight effect

TABLE III

NATURE OF THE SOLUTION	OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
	mgm	
Neutral NaCl + KCl + CaCl	0.58	1.00
50 cc NaCl + KCl + CaCl ₂ + 0.1 cc $\frac{N}{10}$ NaOH	0.58	1.00
50 cc NaCl + KCl + CaCl ₂ + 0.2 cc $\frac{N}{10}$ NaOH	0.53	0.90
50 cc NaCl + KCl + CaCl ₂ + 0.4 cc $\frac{N}{10}$ NaOH	0.60	1.00
50 cc NaCl + KCl + CaCl + 0.8 cc $\frac{N}{10}$ NaOH	0.83	1.40

We next tried the effect of another strong base, namely, tetraethylammoniumhydroxide, on the rate of oxidations in fertilized eggs of *S. purpuratus*. The following table gives the result

TABLE IV

NATURE OF THE SOLUTION	OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
	mgm	
Neutral NaCl + KCl + CaCl	0.45	1.00
50 cc NaCl + KCl + CaCl ₂ + 0.2 cc $\frac{N}{10}$ N(C ₂ H ₅) ₄ OH	0.44	1.00
50 cc NaCl + KCl + CaCl ₂ + 0.4 cc $\frac{N}{10}$ N(C ₂ H ₅) ₄ OH	0.44	1.00
50 cc NaCl + KCl + CaCl ₂ + 0.5 cc $\frac{N}{10}$ N(C ₂ H ₅) ₄ OH	0.47	1.00

The addition of 0.5 cc of $\frac{N}{10}$ N(C₂H₅)₄OH to 50 cc of salt solution does not influence the rate of oxidation in the fertilized eggs of *S. purpuratus*. The addition of more N(C₂H₅)₄OH would interfere too much with the analytical result and was not undertaken.

2 Weak bases influence the rate of oxidations in the fertilized egg of *Strongylocentrotus purpuratus* but slightly. This is shown in table V for NH₄OH and in table VI for methylamine.

This result agrees with the observation of Warburg.

3 We have to explain two groups of facts.

1 The apparent difference in the action of weak and strong bases on fertilized eggs.

2 The difference in the reaction of fertilized and unfertilized eggs. We will take up these questions in succession. The fact

TABLE V

NATURE OF THE SOLUTION	COEFFICIENT OF RATE OF OXIDATIONS	
	OXYGEN CONSUMED	
	mgm	
Neutral NaCl + KCl + CaCl ₂ { a	0 43	
b	0 47	
50 cc NaCl + KCl + CaCl + 0 6 cc $\frac{N}{10}$ NH ₄ OH	0 66	1 47
50 cc NaCl + KCl + CaCl ₂ + 1 0 cc $\frac{N}{10}$ NH ₄ OH	0 65	1 44
50 cc NaCl + KCl + CaCl ₂ + 1 4 cc $\frac{N}{10}$ NH ₄ OH	0 61	1 36
50 cc NaCl + KCl + CaCl ₂ + 1 6 cc $\frac{N}{10}$ NH ₄ OH	0 59	1 31
50 cc NaCl + KCl + CaCl ₂ + 1 8 cc $\frac{N}{10}$ NH ₄ OH	0 63	1 40
50 cc NaCl + KCl + CaCl ₂ + 2 0 cc $\frac{N}{10}$ NH ₄ OH	0 61	1 36
Neutral NaCl + KCl + CaCl ₂	0 55	
50 cc NaCl + KCl + CaCl ₂ + 0 1 cc $\frac{N}{10}$ NH ₄ OH	0 60	1 09
50 cc NaCl + KCl + CaCl ₂ + 0 3 cc $\frac{N}{10}$ NH ₄ OH	0 74	1 35
50 cc NaCl + KCl + CaCl ₂ + 0 6 cc $\frac{N}{10}$ NH ₄ OH	0 69	1 25
50 cc NaCl + KCl + CaCl ₂ + 1 0 cc $\frac{N}{10}$ NH ₄ OH	0 74	1 35
50 cc NaCl + KCl + CaCl ₂ + 1 8 cc $\frac{N}{10}$ NH ₄ OH	0 70	1 27
50 cc NaCl + KCl + CaCl ₂ + 2 4 cc $\frac{N}{10}$ NH ₄ OH	0 72	1 31
50 cc NaCl + KCl + CaCl ₂ + 3 0 cc $\frac{N}{10}$ NH ₄ OH	0 73	1 33

TABLE VI

NATURE OF THE SOLUTION	COEFFICIENT OF RATE OF OXIDATIONS	
	OXYGEN CONSUMED	
	mgm	
Neutral NaCl + KCl + CaCl	0 41	
50 cc NaCl + KCl + CaCl ₂ + 0 4 cc $\frac{N}{10}$ methyl-amine	0 46	
50 cc NaCl + KCl + CaCl ₂ + 0 6 cc $\frac{N}{10}$ methyl-amine	0 44	
50 cc NaCl + KCl + CaCl ₂ + 0 8 cc $\frac{N}{10}$ methyl-amine	0 40	
Neutral NaCl + KCl + CaCl ₂	0 42	
50 cc NaCl + KCl + CaCl + 1 0 cc $\frac{N}{10}$ methyl-amine	0 34	
50 cc NaCl + KCl + CaCl + 1 2 cc $\frac{N}{10}$ methyl-amine	0 27	eggs have suffered considerably and increasingly
50 cc NaCl + KCl + CaCl ₂ + 1 4 cc $\frac{N}{10}$ methyl-amine	0 26	

that weak bases have no effect upon the rate of oxidations in fertilized eggs of *purpuratus* finds its explanation in the fact that strong bases do not act either except if their concentration is above $10^{-3}N$. If we assume that this effect is due to the concentration of OH ions it would require a concentration of $\frac{N}{1000}$ NH_4OH or more to produce a similar effect, since the degree of dissociation of NH_4OH is so much lower than that of NaOH. Such a concentration would probably destroy the eggs very rapidly.

It seems very important to point out that we cannot draw any conclusion from this experiment upon the seat of normal oxidations in the egg, since that concentration of NaOH required to raise the rate of oxidations in the fertilized egg is injurious. We ascertained the maximum concentration of NaOH which allows the egg to develop normally (in a mixture of NaCl + KCl + $CaCl_2$) and found that it varies somewhat between 0.2 and 0.4 cc of $\frac{N}{10}$ NaOH to 50 cc of solution. The minimal concentration in which NaOH increases the rate of oxidations in the fertilized egg lies above this limit. We are therefore dealing with a kind of injurious (etching?) effect of the NaOH, which is probably in no way related to the normal processes of oxidations. The weaker bases do not produce this injurious (etching?) effect in the concentrations in which they can be applied and this accounts for the fact that in the fertilized eggs an increase in the rate of oxidations can apparently only be produced by strong and not by weak bases.

The second, and perhaps more interesting question, is, why the weak bases influence the rate of oxidations in the unfertilized eggs so powerfully while they are absolutely ineffective in the fertilized egg. There are two possible explanations. First, it may be said that the bases raise the rate of oxidations in the unfertilized egg only indirectly by causing a liquefaction or cytolysis of the cortical layer. This in itself causes, as was shown by Warburg and confirmed by ourselves, a rise in the rate of oxidations, no matter by which means this cytolysis was caused.

It is secondly possible that the cytolysis of the cortical layer by bases is the result of oxidation, since the bases do not induce development in the unfertilized egg if the oxidations are suppressed. If this be true we must assume that the cortical layer contains a substance which can be liquefied by bases directly or indirectly.

sodium hydrate and, after the addition of sodium sulphate and some water, was shaken out with ether repeatedly. The ether was washed several times with sodium carbonate solution and evaporated to dryness. The residue was very small in amount, not crystalline, it looked like oleic acid. It gave no positive tests for cholesterol either by Salkowski's or the Liebermann-Burchard method. I have repeatedly sought for cholesterol in these eggs varying the procedure but I have never been able to find it. On one occasion when the ovaries were not ripe the fatty residue of the ether after repeated saponifications, both with alkali and acid, gave a very faint, transitory green such as cholesterol gives in the Liebermann test, and there may have been a very small amount of cholesterol present, but no crystals could be obtained. In view of the fact that the color reaction is probably not specific I am doubtful whether there was a trace of cholesterol present or not. It could not be positively identified. It may be mentioned that cholesterol in combination as in lanolin gives the Liebermann-Burchard reaction very strongly.

The same methods applied to the sea-urchin egg gave, as usual, a crystalline mass on evaporating the ether after saponification, the crystals looked like cholesterol and gave a typical reaction of Salkowski. I may say that the extract of the whole body of the star-fish contains cholesterol in abundance.

Another very interesting peculiarity of the star-fish egg is the character of its phosphatide. It resembles the jecorn described by Drechsel. A large quantity of eggs was extracted with hot alcohol and ether, the lecithin (?) precipitated from the ether solution in the usual way, redissolved in ether (not anhydrous) and reprecipitated with acetone and the process repeated until it dissolved quite clear in the ether and did not settle out a white substance when standing in the cold. This white substance coming out of the ether had a sweet taste, but had no reducing action on Fehling's solution either before or after heating with hydrochloric acid. The phosphatide thus prepared is more hygroscopic than lecithin from the brain or eggs. It makes unusually beautiful, regular myelin forms when shaken with water, and it seems to be toxic for sea-urchin eggs. It was probably not a pure substance. It contains a large amount of a reducing sugar which, calculated as glucose, amounts to 10.51 per cent by weight. The nature of this

sugar was not determined, an osazone was prepared, the fermentation test was indecisive. The lecithin itself does not reduce Fehling's solution but only after it has been heated with acid. I heated it for ten hours with 3.5 per cent HCl and determined the sugar by the reduction of Fehling's solution according to the method of Munson and Walker. This phosphatide also contains sulphuric acid in an ester form like Koch's sulphatide. It contained in a single analysis 1.19 per cent of sulphur in an oxidized form. This is in organic combination. The fatty acids are very largely oleic, or a similar acid, having an ether-soluble lead salt. The analysis of this impure phosphatide resulted as follows

Glucose (?)	10.51 per cent
Fatty acids	46.16 per cent
Phosphorus	3.57 per cent
Sulphur	1.19 per cent

Of the fatty acid approximately 71.35 per cent was recovered as oleic (?) acid. This phosphatide also contains a considerable amount of magnesium, but I did not determine it quantitatively.

I may mention that, of the total ether-soluble portion of the alcohol-ether extract of these eggs, the lecithin in one case weighed 0.6105 gram, the fat, the part not precipitated by acetone, 0.6055 gram, so that there are about equal quantities of fat and lecithin.

SUMMARY

Cholesterol is either absent altogether or present in very small amount in the star-fish egg. It could not be positively found in the eggs of *Asterias forbesii*. It is present in considerable quantities in the sea-urchin egg. This difference possibly is correlated with the greater sensitiveness to cytolysis of the star-fish egg. The phosphatide of the star-fish contains about 10 per cent of a reducing sugar in firm combination and also sulphuric acid.

THE INFLUENCE OF HYPERTONIC SOLUTION UPON THE RATE OF OXIDATIONS IN FERTILIZED AND UNFERTILIZED EGGS

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I

In a series of papers published since 1905 one of the writers has shown that in the egg of the sea urchin the initiation of normal development requires two different agencies. The one is needed to call forth a typical change in the surface of the egg which results in the formation of a more or less typical "fertilization membrane." This change causes the egg to segment and if the temperature is low some of these eggs may reach an early larval stage. At room temperature, however, the eggs begin to disintegrate after artificial membrane formation, as a rule, during the first cell division. These facts prove that the artificial membrane formation (e g, by butyric acid) suffices to set the whole machinery of cell division and development into motion but that the egg is sickly and disintegrates the more rapidly the higher the temperature.¹

It was shown by the same author that this disintegration after artificial membrane formation is retarded or suppressed if we deprive the egg of oxygen or if we retard the rate of oxidations in the egg through the addition of a trace of KCN. The addition of a sufficient amount of chloral hydrate (or probably any other narcotic) acts in the same way, although chloral hydrate does not lower the rate of oxidations. But all these agencies have one object in common, namely, that they inhibit the processes of nuclear and cell division. The disintegration of the unfertilized

¹ Loeb *Die chemische Entwicklungserregung des tierischen Eies*, Berlin 1909, *The Mechanistic Conception of Life*, 1912

egg after membrane formation is therefore connected either with oxidations or with the nuclear and cell divisions consequent upon oxidations. We can cure the egg from this disease by one of two agencies. We either put it for a short time (from 30 to 60 minutes) into a neutral hypertonic solution, or we put it for a longer time (about 3 hours) into sea water which is free from oxygen or which contains some KCN. The former method gives more uniform results. Such eggs develop into larvae at room temperature.

These results gained in importance since it could be shown that the developmental effect of the spermatozoon is also due to two different agencies, one of which causes merely membrane formation while the other produces the corrective effect.²

Warburg had already found that the artificial membrane formation raises the rate of oxidations in the egg to the same height as the entrance of the spermatozoon, as was to be expected and as we were able to confirm.³ Since Loeb had found that the hypertonic solution acts only in the presence of free oxygen and that its action is suppressed by the addition of a trace of KCN, it was of interest to find out whether or not the hypertonic solution alters the rate of oxidations in the egg after artificial membrane formation. The experiments were made in this way, that the rate of oxidations in the eggs, after artificial membrane formation, was determined, first in normal sea water and later in hypertonic solution.

These experiments are by no means simple, since it is necessary that all the eggs possess membranes, for we shall see afterwards that in unfertilized eggs without membranes the hypertonic solution causes a decided rise in the rate of oxidations.

The unfertilized eggs of one female, *Strongylocentrotus purpuratus*, were divided into six equal parts. Two remained unaltered and served as checks. The eggs of two lots were treated with butyric acid and all formed membranes. One of these lots was put into normal sea water, the other into hypertonic sea water (50 cc sea water + 8 cc $\frac{5M}{2}$ NaCl) such as is used to cause the eggs to develop normally after artificial membrane formation. The fifth lot was fertilized with sperm. The sixth lot served for some other experiment which does not concern us here. The eggs remained in each solution one hour. Temperature 18°C.

² Loeb *loc cit*

³ Warburg *Zeitschr f physiol Chem*, lxxvi, p 305, 1910

TABLE I

	OXYGEN CONSUMED	COEFFICIENT OF OXIDATIONS
	mgm	
1 Unfertilized eggs in normal sea water (control 1)	0 12	1 00
2 Unfertilized eggs in normal sea water (control 2)	0 12	1 00
3 Unfertilized eggs after membrane formation in normal sea water	0 53	4 40
4 Unfertilized eggs after membrane formation in hypertonic sea water	0 54	4 50
5 Fertilized eggs in normal sea water	0 57	4 70

It is obvious that the hypertonic solution does not increase the rate of oxidations in the unfertilized eggs after artificial membrane formation Table II gives the result of a second experiment of the same kind Temperature 15°C

TABLE II

	OXYGEN CONSUMED PER HOUR	COEFFICIENT OF OXIDATIONS
	mgm	
1 Unfertilized eggs in normal sea water	0 18	1 00
2 Unfertilized eggs after membrane formation in normal sea water	0 85	4 72
3 Unfertilized eggs after membrane formation in hypertonic sea water	0 88	4 88
4 Fertilized eggs in normal sea water	0 82	4 55

The result is identical with that in table I Membrane formation raises the rate of oxidations to the same height as fertilization, but the subsequent treatment of these eggs with the hypertonic solution has no effect upon the rate of oxidations This experiment was repeated three times with the same results as shown in Table III The oxygen consumption is always for one hour at 18°C

All these experiments prove conclusively that the curative effect of the hypertonic solution after the artificial membrane formation is not due to an increase in the rate of oxidations in the egg It cannot be said, however, that it is independent of oxidations,

TABLE III

NUMBER OF EXPERIMENT		OXYGEN CONSUMED PER HOUR	COEFFICIENT OF OXIDATIONS
		<i>mgm</i>	
1	Unfertilized eggs after membrane formation in normal sea water	0 83	1 00
	Unfertilized eggs after membrane formation in hypertonic sea water	0 74	0 90
2	Unfertilized eggs after membrane formation in normal sea water	0 52	1 00
	Unfertilized eggs after membrane formation in hypertonic sea water	0 54	1 04
3	Unfertilized eggs after membrane formation in normal sea water	0 74	1 00
	Unfertilized eggs after membrane formation in hypertonic sea water	0 70	0 90

since the curative effect of the hypertonic solution is retarded or suppressed if the oxidations in the egg are suppressed Loeb formerly suggested that through the exposure to the hypertonic solution an oxidation product is formed in the egg whereby the latter is saved from the threatening disintegration. It may be that an injurious substance contained in the egg after membrane formation is destroyed or that a new substance lacking in the egg is supplied. The same curative effect can be produced more slowly through other processes in the egg which take place in the absence of oxygen.

II THE INFLUENCE OF HYPERTONIC SOLUTIONS UPON THE RATE OF OXIDATIONS IN FERTILIZED EGGS

Since the unfertilized egg after artificial membrane formation behaves in regard to oxidations like a fertilized egg, it was of interest to find out whether or not the hypertonic solution accelerates the rate of oxidations in eggs fertilized by sperm. O. Warburg states that this is the case, and that the increase may be 300 per cent.⁴ We absolutely failed to notice any increase in the rate of oxidations when the eggs of *S. purpuratus* were put into hypertonic sea water, no matter how great the degree of hypertonicity. In

⁴ Warburg *Zeitschr f physiol Chem* ix, p 442, 1909

part of the experiments, 1-3, the rate of oxidations was successively measured in the same eggs for one and a half hours in normal sea water, and then for one hour in the hypertonic sea water. In the experiments 4-7 the eggs of the same female were divided into eight equal parts, four of these were put into normal sea water, the others into hypertonic sea water of different concentrations. This experiment may incidentally also serve as a check for the accuracy of the method.

The temperature was 18°C and the consumption of oxygen measured for one and a half hours. Table IV gives the results.

There can be no doubt about the fact that the hypertonic solution does not increase the rate of oxidations in the fertilized egg of *Stonylocentrotus purpuratus*. This harmonizes with our previous

TABLE IV

NUMBER OF EXPERIMENT		OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
		mgm	
1	Fertilized eggs in normal sea water	0 87	1 00
	Fertilized eggs in 50 cc normal sea water + 8 cc $\frac{5M}{2}$ NaCl	0 86	0 99
2	Fertilized eggs in normal sea water	0 60	1 00
	Fertilized eggs in 50 cc normal sea water + 8 cc $\frac{5M}{2}$ NaCl	0 52	0 87
3	Fertilized eggs in normal sea water	0 55	1 00
	Fertilized eggs in 50 cc normal sea water + 8 cc $\frac{5M}{2}$ NaCl	0 59	1 07
4	Fertilized eggs in normal sea water	1 30	1 00
	Fertilized eggs in 50 cc normal sea water + 4 cc $\frac{5M}{2}$ NaCl	1 27	0 98
5	Fertilized eggs in normal sea water	1 30	1 00
	Fertilized eggs in 50 cc normal sea water + 12 cc $\frac{5M}{2}$ NaCl	1 54	1 20
6	Fertilized eggs in normal sea water	1 33	1 00
	Fertilized eggs in 50 cc normal sea water + 12 cc $\frac{5M}{2}$ NaCl	1 53	1 20
7	Fertilized eggs in normal sea water	1 33	1 00
	Fertilized eggs in 50 cc normal sea water + 16 cc $\frac{5M}{2}$ NaCl	1 57	1 20

result that the hypertonic solution does not increase the rate of oxidations in unfertilized eggs of the same species after artificial membrane formation, since the membrane formation is the essential feature in the causation of development by sperm or by artificial means

III

The fact that a hypertonic solution does not increase the rate of oxidations in the unfertilized eggs after artificial membrane formation seems at first sight to contradict an observation made by Warburg at Naples, that the hypertonic solution raised the rate of oxidations in unfertilized eggs (which have not been submitted to the process of membrane formation) Warburg found that the hypertonic solution raises the rate of oxidations in such eggs as much as ten times⁵ We repeated these experiments on the eggs of *Strongylocentrotus purpuratus* at Pacific Grove, and were able to confirm Warburg's results although the rise in the rate of oxidations was much smaller than that observed in his experiments In our experiments the rate of oxidations was first determined for one and a half hours in normal sea water and then for one and a half hours in hypertonic sea water Table V on the following page gives the results The temperature was 18°C

It should be pointed out that experiments 2 and 3, and 4 and 5, are made on equal lots of eggs The results may serve as a check for the accuracy of the method

The question arises Why is it that the hypertonic solution causes a rise in the rate of oxidations in the unfertilized egg without membrane formation, while it has no such effect on the same eggs after membrane formation? The answer is that the hypertonic solution can cause the membrane formation and that it only raises the rate of oxidations in those eggs in which it causes membrane formation In Loeb's original method of causing artificial parthenogenesis by merely putting the eggs into a hypertonic solution, the hypertonic solution had two kinds of effects it caused first the membrane formation (or the formation of a gelatinous surface film) and at the same time furnished the curative effect

⁵ Warburg *Zeitschr f physiol Chem*, lx, p 443, 1909

TABLE V

NUMBER OF EXPERIMENT	UNFERTILIZED EGGS WITHOUT MEMBRANES IN	OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
		<i>mgm</i>	
1	Normal sea water	0 45	1 00
	Hypertonic (50 cc sea water + 4 cc $\frac{5M}{2}$ NaCl)	0 65	1 40
2	Normal sea water	0 50	1 00
	Hypertonic (50 cc sea water + 6 cc $\frac{5M}{2}$ NaCl)	0 92	1 80
3	Normal sea water	0 49	1 00
	Hypertonic (50 cc sea water + 6 cc $\frac{5M}{2}$ NaCl)	0 84	1 70
4	Normal sea water	0 33	1 00
	Hypertonic (50 cc sea water + 6 cc $\frac{5M}{2}$ NaCl)	0 73	2 20
5	Normal sea water	0 35	1 00
	Hypertonic (50 cc sea water + 8 cc $\frac{5M}{2}$ NaCl)	0 73	2 50
6	Normal sea water	0 46	1 00
	Hypertonic (50 cc sea water + 8 cc $\frac{5M}{2}$ NaCl)	1 19	2 60
7	Normal sea water	0 48	1 00
	Hypertonic (50 cc sea water + 9 cc $\frac{5M}{2}$ NaCl)	1 23	2 60
8	Normal sea water	0 29	1 00
	Hypertonic (50 cc sea water + 9 cc $\frac{5M}{2}$ NaCl)	0 90	3 10
9	Normal sea water	0 30	1 00
	Hypertonic (50 cc sea water + 9 cc $\frac{5M}{2}$ NaCl)	0 67	2 20
10	Normal sea water	0 56	1 00
	Hypertonic (50 cc sea water + 12 cc $\frac{5M}{2}$ NaCl)	1 29	2 30
11	Normal sea water	0 38	1 00
	Hypertonic (50 cc sea water + 16 cc $\frac{5M}{2}$ NaCl)	0 97	2 60

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That the hypertonic solution can cause a cytolytic effect upon the cortical layer of the egg was shown by experiments on the eggs of *Lotha* (a mollusc) whose chorion can be liquefied by hypertonic solutions as well as by bases, if free oxygen is present ⁶

That the two agencies, the membrane-forming and the corrective one, can act simultaneously is not surprising. The same fact can be shown for bases. It is immaterial whether the base is applied first and is then followed by the corrective action of the hypertonic solution or whether both agencies are applied simultaneously.

In our experiments the effect of the hypertonic solution upon oxidations was smaller than that caused by membrane formation with butyric acid. In the latter case, the rate of oxidations was raised from four to six times, while the hypertonic solution, when acting upon unfertilized eggs without membrane formation, raised the rate as a rule to not more than two and one-half and at the utmost three times the amount observed in the same eggs in normal sea water. This difference can be accounted for either by the fact that an exposure of one and a half hours of the eggs of *S. purpuratus* to hypertonic sea water as a rule does not suffice to bring about membrane formation and development, or by the fact that these experiments were made early in the season when fertilization did not raise the rate of oxidations as much as it did later in the season.

We repeated these experiments later in the season to find out whether the rate of oxidations would increase if the eggs remained a longer time in the hypertonic sea water. The temperature was 18°C. The consumption of oxygen was determined for each hour.

TABLE VI.

UNFERTILIZED EGGS IN	OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
	<i>mgm</i>	
Normal sea water	0 16	1 00
Hypertonic sea water, 1st hour	0 67	4 18
Hypertonic sea water, 2d hour	0 79	4 94
Hypertonic sea water, 3d hour	0 64	4 00
Hypertonic sea water, 4th hour	0 56	3 55
Hypertonic sea water, 5th hour	0 51	3 18

⁶ Loeb *Univ of Calif Publ Physiol*, III, 1905

A repetition of the experiment gave a similar result. In this case the hypertonic solution had the same effect upon the rate of oxidations as fertilization or membrane formation. It may also be that by chance we are dealing in this case with the eggs of a female which were very susceptible to the treatment with hypertonic solution while in the previous experiments the eggs had been more or less refractory. As we stated, not the eggs of every female of *purpuratus* develop if treated with hypertonic sea water.

These results support also the idea that the rise in the rate of oxidations is due to the membrane-forming action of the hypertonic solution, which of course takes place during the first two hours. We see that the maximal increase in the rate of oxidations takes place during that time.

IV

If it is true that the increase in the rate of oxidations observed in these cases is merely due to the membrane-forming effect of the hypertonic solution, we should expect that if we add a weak base to the hypertonic solution, the increase in the rate of oxidations should be no greater than that caused by the weak base alone, the reason being that the weak base alone causes the membrane formation.⁷ This reasoning is supported by facts. The rate of oxidations was compared in unfertilized eggs (without membranes) in alkaline sea water and hypertonic sea water to which the same amount of base had been added. The hypertonic sea water consisted of 50 cc sea water + 8 cc $\frac{5M}{2}$ NaCl + KCl + CaCl₂. The eggs were distributed into equal portions. One-half was put into 50 cc of sea water + 1 cc of the base, the other half of the eggs was first put into normal sea water and then into 50 cc of hypertonic sea water + 1 cc of the same base. Time of exposure one and a half hours, temperature 18°C.

⁷ Loeb *Journ of Exp Zoology*, xiii, p 577, 1912

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TABLE VII

NUMBER OF EXPERIMENT	UNFERTILIZED EGGS (WITHOUT MEMBRANES) IN	OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
		mgm	
1	Normal sea water	0 22	1 00
	50 cc hypertonic sea water + 1 cc $\frac{N}{10}$ NH_4OH	1 20	5 40
	50 cc normal sea water + 1 cc $\frac{N}{10}$ NH_4OH	0 88	4 00
2	Normal sea water	0 37	1 00
	50 cc hypertonic sea water + 1 cc $\frac{N}{10}$ benzylamine	1 89	5 10
	50 cc normal sea water + 1 cc $\frac{N}{10}$ benzylamine	1 75	4 70
3	Normal sea water	0 36	1 00
	50 cc hypertonic sea water + 1 cc $\frac{N}{10}$ butylamine	1 73	4 80
	50 cc normal sea water + 1 cc $\frac{N}{10}$ butylamine	1 67	4 60

It is obvious that the weak base alone raised the rate of oxidations practically to the same height as the combination of base and hypertonic sea water. The whole rise was due to the membrane-forming effect for which the weak base was sufficient.

In the case of a strong base, the result may be different, since neither the base nor the hypertonic solution alone may cause membrane formation (or the change in the cortical layer of the egg) necessary for development. The following may serve as an example. Duration of experiment one and a half hours, temperature 18°C.

TABLE VIII

UNFERTILIZED EGGS (WITHOUT MEMBRANE FORMATION) IN	OXYGEN CONSUMED	COEFFICIENT OF RATE OF OXIDATIONS
	mgm	
Normal sea water	0 41	1 00
50 cc hypertonic sea water + 1 cc $\frac{N}{10}$ NaOH	0 81	2 00
50 cc normal sea water + 1 cc $\frac{N}{10}$ NaOH	0 46	1 20

In this case the NaOH had little effect and hence the hypertonicity caused a noticeable increase in the rate since it probably increased the number of eggs in which the process of membrane formation was started.

V

Finally, we wish to report an experiment which does not strictly belong here but which shows that the mere cytolysis of the cortical layer is responsible for the increase in the rate of oxidations observed after membrane formation either by a spermatozoon or by butyric acid. It is possible to cause complete cytolysis of the unfertilized egg of *S. purpuratus* with saponin and we found that this increases the rate of oxidations to the same extent as fertilization by sperm. The following may serve as an example. Unfertilized eggs of *S. purpuratus* were used, temperature 15°C

TABLE IX

	OXYGEN CONSUMED PER HOUR	COEFFICIENT OF RATE OF OXIDATIONS
	<i>mgm</i>	
Unfertilized eggs	0 15	1 00
The same eggs after cytolysis with saponin	1 07	7 10
Unfertilized eggs	0 22	1 00
The same eggs after cytolysis with saponin	0 80	3 60

The variation in the effect of cytolysis in the two experiments may be due to the fact that in the second experiment an excessive amount of saponin was used.

This experiment proves that the increase in the rate of oxidations due to fertilization or artificial membrane formation is merely caused by the cytolysis of the cortical layer.

VI THEORETICAL REMARKS

It seems that all the experiments point very clearly towards one conclusion, namely, that the hypertonic solution raises the rate of oxidations in the unfertilized eggs of *S. purpuratus* only under one condition, *i e*, if it causes the change at the surface of the egg underlying membrane formation. In eggs which have undergone the process of membrane formation either by fertilization or by a treatment with butyric acid, the hypertonic solution causes no further increase in the rate of oxidations.

SUMMARY

1 The unfertilized eggs of sea urchins which have undergone artificial membrane formation die if not treated with a hypertonic solution. It is shown in these experiments that the rate of oxidations in such eggs is not increased by the hypertonic solution.

2 It is shown that the hypertonic solution does not cause an increase in the rate of oxidations of fertilized eggs of *Strongylocentrotus purpuratus*.

3 Hypertonic solutions increase the rate of oxidations in unfertilized eggs which have not undergone the process of membrane formation, as Warburg observed. This increase is purely due to the fact that the hypertonic solution induces the change in the cortical layer of the egg which leads to membrane formation.

4 This conclusion is supported by the fact shown in this paper, that the addition of a weak base to normal sea water, which causes development (or membrane formation) increases the rate of oxidations in unfertilized eggs to the same amount as if it were added to hypertonic sea water. Since in this case the membrane-forming effect can be produced by the base alone the addition of the hypertonic solution can add nothing to the effect.

5 Complete cytolysis of the unfertilized egg by saponin raises the rate of oxidations to the same height as fertilization, thus showing that the cytolysis of the cortical layer of the egg is the essential feature in fertilization.

DO GLIADIN AND ZEIN YIELD LYSINE ON HYDROLYSIS?¹

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GLIADIN

Kossel and Kutscher² failed to find any lysine in the alcohol-soluble proteins of maize or wheat flour, and their experience has been confirmed by others³ It has since been generally assumed that alcohol-soluble proteins lack the lysine complex, and consequently gliadin and zein have been used in experiments, from the outcome of which conclusions have been drawn respecting the part played by lysine in nutrition, especially in connection with the synthesis of amino-acids by the animal organism⁴ Observations have been recorded, however, which have shown that preparations of gliadin may sometimes yield lysine Thus Abderhalden and Funk⁵ in discussing Henriques' experiments state that unless gliadin preparations are very carefully purified they always yield small amounts of lysine They do not, however, say how they detected the lysine nor how the preparations should be purified

Van Slyke⁶ by aid of his method for determining the distribution of nitrogen in proteins found in gliadin a quantity of nitrogen

¹ The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington

² Kossel and Kutscher *Zeitschr f physiol Chem*, **xxxi**, p 165, 1900

³ Abderhalden and Samuely *ibid*, **xliv**, p 276, 1905, Osborne and Clapp *Amer Journ of Physiol*, **xvii**, p 231, 1906, Ackermann *Zeitschr f physiol Chem*, **lxiv**, p 91, 1910

⁴ Michaud *Zeitschr f physiol Chem*, **lx**, p 405, 1909, Henriques *ibid*, **lx**, p 105, 1909, Osborne and Mendel *this Journal*, **xii**, p 473, 1912

⁵ Abderhalden and Funk *Zeitschr f physiol Chem*, **lx**, p 418 1909

⁶ Van Slyke *this Journal*, **x** p 15, 1911

in the lysine fraction corresponding to 0.75 per cent of lysine. He considers this small amount to fall within the limit of experimental error and that it is not to be regarded as contradictory to the negative results of previous authors. Kossel and Gawrilow⁷ found that 0.8–0.4 per cent of the total nitrogen of hordenin, equal to about 2 per cent of lysine, reacts with formaldehyde, whereas none of the nitrogen of zein reacts therewith. This observation shows that hordenin may contain a small proportion of lysine, for there is reason to believe that lysine furnishes the only free amino groups present in most proteins.

In a recent paper⁸ from this laboratory attention was called to the fact that a preparation of gliadin, which had been made with care, and was supposedly as pure as preparations of this protein can reasonably be made, yielded a very small amount of lysine when hydrolyzed by long boiling with sulphuric acid. The question was then left open whether this lysine originated from a contamination of the preparation or from the gliadin itself.

Since it has become a matter of importance to know whether or not gliadin prepared by the usual methods is wholly free from lysine we have subjected the preparation previously examined to a rigid fractional precipitation from alcoholic solution, and have again detected lysine in each of the extreme fractions, and in approximately the same proportion as that previously found in the original preparation. No evidence was secured which indicated the presence of glutenin, or any other protein in this preparation, and as the same relatively very high proportion of nitrogen as ammonia, and low proportion of basic nitrogen, which characterize gliadin, were found in the two extreme fractions and in the original preparation we are inclined to think that this preparation contained no other protein than gliadin, and that gliadin does in fact yield so little lysine that its presence in purified preparations has heretofore escaped detection by the usual methods of analysis. The amount of lysine in the original preparation was so small that in following Kossel's method no precipitate was found when the alcoholic solution of picric acid was added to the properly concentrated alcoholic solution of the amino-acids pre-

⁷ Kossel and Gawrilow *Zeitschr f physiol Chem*, lxxxii, p 274, 1912

⁸ Osborne and Mendel *this Journal*, xii, p 473, 1912

precipitated by phosphotungstic acid, after the arginine and histidine had been precipitated by silver nitrate and baryta

By allowing the alcoholic solution containing the picric acid to evaporate slowly a semi-crystalline residue remained which was extracted with alcohol. The part which was not dissolved was recrystallized from water, yielding a product which appeared in all respects like lysine picrate. This darkened on heating at about 225° and exploded at 256° in the manner characteristic of lysine picrate. When mixed with pure lysine picrate the behavior on heating was unchanged. The nitrogen content found by digesting with sulphuric and salicylic acids and zinc dust, and then boiling for several hours, was 18.34 per cent. Calculated for lysine picrate, 18.68 per cent.

Since large quantities of alcohol were used in extracting the wheat gluten, from which this gliadin was obtained, it was possible that the lysine thus found came from a contamination with glutenin or some other protein present in the original gluten. If this were so the greater part of such contamination should appear as an insoluble residue when the preparation was dissolved again in a limited quantity of 70 per cent alcohol. We accordingly treated 500 grams of the gliadin with 2500 cc. of 70 per cent (by volume) alcohol and obtained a slightly turbid solution which on long standing deposited a very small amount of insoluble matter. By repeatedly washing this sediment by decantation with 70 per cent alcohol only an insignificant quantity remained, from which we conclude that this preparation of gliadin contained, at the most, but traces of glutenin, or other protein insoluble in dilute alcohol.

The somewhat opalescent alcoholic solution deposited nothing more on standing or centrifugalization, and became perfectly clear on warming slightly. In order to find whether or not this solution could be separated by fractional precipitation into products indicating the presence of two or more proteins, of which one might contain the lysine, we treated the solution as follows.

The entire solution, of 3 liters' volume, was mixed with 3 liters of 80 per cent alcohol, by pouring the two simultaneously into a large jar, and stirring rapidly during the mixing, so as to avoid precipitating any gliadin by locally raising the concentration of the alcohol above 75 per cent through imperfect mixing. A turbid solution resulted which showed no sign of a precipitate. This

solution was then mixed, in the same way, with 6 liters of 85 per cent alcohol, whereupon a flocculent precipitate separated from the solution, now containing 80 per cent of alcohol. After stirring violently the precipitate separated in flocks and soon settled, leaving the solution nearly clear. In this way any fraction less soluble in the more concentrated alcohol should be separated from that soluble therein, for the increased volume of the stronger alcohol could be expected to keep the latter in solution.

After the precipitate had settled it became coherent, whereupon the nearly clear solution was decanted very completely. The precipitate was then treated with about 1500 cc of 80 per cent alcohol, by which little of it was dissolved. It was thereby converted into a voluminous, snow-white, flocculent substance which differed in its physical character from the gliadin precipitates usually obtained by pouring gliadin solutions into strong alcohol. This was separated from the 80 per cent alcoholic washings by centrifugalization, suspended in 80 per cent alcohol and then left for about forty hours to settle. The colloidal suspension which remained was treated with a few drops of a solution of ammonium acetate, dissolved in 80 per cent alcohol, which caused a practically complete separation, leaving the alcohol nearly clear, and rendered the precipitate glutinous. The precipitate was then extracted with 70 per cent alcohol, the undissolved residue centrifugated out, dehydrated by absolute alcohol, and dried over sulphuric acid. This product, *A*, which weighed 55 grams, should contain whatever glutenin was present in the original preparation, any gliadin that had been altered and rendered less soluble by the processes employed in making the original preparation, any contaminating protein sparingly soluble in strong alcohol, or, if gliadin is a mixture of proteins, as some have supposed, more or less of any constituent of such a mixture which was less soluble in stronger alcohol than the rest.

The 12 liters of 80 per cent alcohol, from which *A* had been separated, after standing several days, gradually yielded a small, semi-fluid, transparent deposit from which the solution was decanted. This deposit was dissolved completely and readily by cold 70 per cent alcohol, its solution united with that obtained by digesting *A* with 70 per cent alcohol, concentrated to a syrup on a steam bath, and then poured into a large volume of absolute

alcohol The resulting precipitate, after digesting with absolute alcohol and ether, was freed from the latter in a desiccator over sulphuric acid This fraction, *B*, weighed 38 grams

The 12 liters of 80 per cent alcoholic solution, together with the washings of the same strength of alcohol, 3 liters, were united, concentrated in a vacuum to about 1500 cc and then on a steam bath to a syrup which was finally poured into a large volume of absolute alcohol The precipitate thus produced was dehydrated by digesting with absolute alcohol, washed with ether, and freed from alcohol and ether in a desiccator over sulphuric acid This fraction, *C*, weighed 298 grams

The original gliadin preparation was thus divided into three fractions, *A*, insoluble in 70 per cent alcohol, *B*, insoluble in 80 per cent alcohol but soluble in 70 per cent, and *C*, soluble in 80 per cent alcohol

In order to detect differences between the extreme fractions *A* and *C*, the following analytical data were secured which are given as per cent of the protein

PREPARATION		NH ₂ N	BASIC N	ARGININE	HISTIDINE	LYSINE	TYROSINE
<i>A</i>	I	4.66	0.76	2.48	1.43	0.15	1.39
	II	4.66	0.76	2.43	1.44		
<i>C</i>	I	4.39	1.01	2.92	1.49	0.07	1.50
	II	4.39	1.01	2.90	1.48		
Original	I			2.67	1.63	0.16	1.54
	II			2.78	1.49		1.68

From fractions *A* and *C* lysine was isolated in the same way as from the original preparation No precipitate of lysine picrate was obtained by adding an alcoholic solution of picric acid to the suitably concentrated alcoholic solution of the amino-acids precipitated by phosphotungstic acid, and it was only by persistent effort that any lysine picrate was obtained from either solution The identity of the picrate was established by the decomposition point, which was unchanged on mixing with pure lysine picrate, and also by the nitrogen content

If the probable limits of accuracy of the methods employed in making the above determinations are considered, the results

obtained afford no basis for concluding that the original preparation has been separated into two chemically different parts. The only difference worthy of consideration is that between the ammonia nitrogen of *A* and *C*. This difference is distinctly beyond the limits of the analytical method, but it must be recalled that a little ammonium acetate solution was used to cause the colloidal suspension of *A* to separate from the final 80 per cent alcoholic wash solution. It is consequently not improbable that some ammonia was combined with, or adsorbed by, the protein and thus contributed to the higher result of this determination.

It is thus evident that the lysine found in the original preparation did not come from a contamination with glutenin, for this protein contains less ammonia nitrogen, and more basic nitrogen and also more arginine than either of the fractions *A* or *C*.

It is also to be noted that lysine was found in both *A* and *C* and since no importance can be attached to the small difference between the quantities found we have no evidence that the original preparation consisted of a lysine-yielding and a lysine-free protein. The fact that the original preparation of gliadin was separated into three fractions which differed in solubility in alcohol of various strengths might be considered as evidence of the presence of more than one protein in the original preparation. Such evidence, however, has no force, for, in the process of isolation, nearly all proteins are liable to slight alterations whereby more or less of them is converted into insoluble products. Furthermore, preparations of proteins made by the only methods now available consist to a greater or less extent of salts of these proteins which differ in solubility from the free protein, as well as from one another. Such differences as here appear cannot, therefore, be accepted as good evidence of the presence of more than one protein in the original preparation of gliadin.

The discovery of this small proportion of lysine in these preparations of gliadin shows how cautious we must be in concluding that any amino-acid is wholly lacking among the products of hydrolysis of a protein when the only evidence for such a conclusion is based on the failure to isolate it by direct crystallization either in the free state or as a crystalline compound with an added reagent.

ZEIN

Having thus found lysine in a supposedly pure preparation of gliadin we made a careful examination of zein employing the same method for obtaining traces of lysine picrate which had proved successful with gliadin. Our long-continued and persistent efforts resulted only in crystalline picrates which decomposed at 272° and proved to be sodium picrate. No trace of lysine picrate was found. The sodium was derived from traces of this base in the large quantities of phosphotungstic acid and baryta used in the analysis.

THE INFLUENCE OF FEVER ON THE ELIMINATION OF CREATININE

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(Received for publication, April 26, 1913)

It has long been recognized that the rate of body metabolism is intensified in the various febrile diseases, resulting in an increased elimination of the nitrogenous waste products in the urine. The study of the urine under these conditions has been of considerable service in the interpretation of intermediary processes which take place in the body during this disturbed state. Metabolism in fever, especially in typhoid fever and pneumonia, has recently received extensive consideration at the hands of a number of investigators, viz, van Hoogenhuyze and Verploegh,¹ Klercker,² Ewing and Wolf,³ Shaffer and Coleman,⁴ and Wolf and Lambert.⁵

That the elimination of total nitrogen and of its chief constituent, urea nitrogen, paralleled the rise in body temperature in various acute infections was noted long ago. The excretion of ammonia was found to be increased by some of the older investigators, though its excretion as determined with more reliable methods would indicate that the ammonia is not ordinarily greatly increased in fever, and further, that acidosis is not a prominent feature of this condition. Uric acid has quite generally been found to be increased during the height of fever, followed usually by a decline with the fall in temperature. Fever was likewise claimed by the older observers to increase the elimination of creatinine, though

¹ van Hoogenhuyze and Verploegh *Zeitschr f physiol Chem*, lvi, p 161, 1908

- Klercker *Zeitschr f klin Med*, lxxviii, p 22, 1909

³ Ewing and Wolf *Arch of Int Med*, iv, p 330, 1909

⁴ Shaffer and Coleman *ibid*, iv, p 538, 1909

⁵ Wolf and Lambert *ibid*, v, p 406, 1910

the data on this point can hardly be regarded as conclusive, owing to the unreliability of the methods available

Since Folin⁶ with his colorimetric method first demonstrated the absolute constancy in the elimination of creatinine by normal individuals under a variety of dietary conditions, this nitrogenous urinary constituent, which stands next to urea in point of quantity, has been a subject of study at the hands of numerous investigators. A lowered creatinine output has been observed to be associated with a variety of diseases,⁷ but the only condition which has been found to be accompanied by an immediate change in the elimination of creatinine is fever, and here the excretion of this constituent is increased, the amount of the increase apparently bearing a close relationship to the rise in body temperature. van Hoogenhuyze and Verploegh⁸ claim to have observed a slightly increased elimination of creatinine after stimulation with syrup of cola and strychnine, and a slight decrease after potassium bromide and absolute rest. The changes they observed were very slight and in no way comparable to the greatly increased elimination during fever. In view of the lack of knowledge with regard to the origin of creatinine, the increased excretion brought about by fever is of particular interest.

This rise in creatinine elimination during fever appears to have been first observed by Leathes⁹ after the production of a hyperpyrexia in normal subjects by the use of antityphoid vaccine. He found that the curve of the creatinine elimination followed both the temperature and the total nitrogen, but that the percentage of creatinine nitrogen in terms of total nitrogen fell somewhat below the normal percentage with a marked increase in the elimination of total nitrogen, though the absolute amount of the creatinine excretion was increased about 25 per cent at the highest point. Subsequently, van Hoogenhuyze and Verploegh¹⁰ noted that the maximum creatinine excretion appeared at the same time or within a few hours of the highest temperature. The observations of Klercker¹¹ on the elimination of creatinine and creatine in febrile

⁶ Folin *Amer Journ of Physiol*, **xii**, p 66, 1905

⁷ Cf Myers *Amer Journ of Med Sci*, **cxxxix**, p 256, 1910

⁸ *Loc cit*

⁹ Leathes *Journ of Physiol*, **xxxv**, p 205, 1907

¹⁰ *Loc cit*

¹¹ *Loc cit*

diseases, but more especially the contributions of Ewing and Wolf¹² and Shaffer and Coleman¹³ on protein metabolism during typhoid fever, and Wolf and Lambert¹⁴ on protein metabolism in pneumonia, have greatly amplified our knowledge on this subject. The increased excretion of creatinine during fever has likewise been noted by Manuchin,¹⁵ though the deductions he draws would hardly appear in accord with other observations.

Not only has an increased excretion of creatinine been observed during the active stages of fever, but a decrease in its elimination during convalescence. It has further been noted that sooner or later during the course of a fever creatine appears in the urine, and its elimination in increased quantities seems of grave diagnostic importance. This was strikingly shown in the series of fatal pneumonias reported by Wolf and Lambert. In general, a parallel between the febrile temperature, total nitrogen, urea and creatinine has been observed. Shaffer and Coleman found that the increased excretion of creatinine in fevers was hardly in proportion to the amount of body protein catabolized, their highest increase in the creatinine excretion being calculated as about 20 per cent. The destruction of body proteins during fever, though perhaps more severe, is quite comparable to that occurring during fasting, and Mendel and Rose,¹⁶ and Myers and Fine¹⁷ have observed that the total creatinine nitrogen (from both creatinine and creatine) forms a uniform proportion of the total nitrogen in starving rabbits.

In producing this increased metabolism in fever, as shown by the increased excretion of nitrogenous waste products and also by the increased elimination of carbon dioxide, several factors play a part, viz, the hyperthermia *per se*, the accompanying inanition, and the toxic agent causing the fever.

That pyrexia, produced by artificially raising the body temperature with a hot bath, or otherwise, will raise the level of body metabolism has been demonstrated. The carbon-dioxide elimination has been observed to be increased from 30 to 40 per cent under these conditions, and somewhat similar figures have been obtained

¹ *Loc cit*

¹³ *Loc cit*

¹⁴ *Loc cit*

¹⁵ Manuchin *Russk. Wratsch*, 18, pp 18, 55, 89, 1910

¹⁶ Mendel and Rose *this Journal*, 1, p 213, 1911

¹⁷ Myers and Fine unpublished data

for the nitrogen elimination Linser and Schmid¹⁸ have pointed out that in man it is not until the body temperature is increased above 39°C that there is an increase in protein metabolism From this Krehl¹⁹ concludes that in infective fevers with a temperature under 40°C the additional protein metabolism must be of toxic origin

In infective fevers, whether in man or animals, it has often been noted that the rise in nitrogenous metabolism is greater than should be observed if due entirely to the pyrexia and accompanying inanition This added excess in the nitrogen elimination has been explained as toxic in origin Ewing and Wolf in their study of protein metabolism in typhoid fever observed that the highest excretion of nitrogen in proportion to body weight occurred in the so-called toxic cases and that, although this was generally coincident with a high temperature, the total nitrogen appeared to be less affected by the fever than by the intoxication Some of the most widely quoted experiments on fever are those by May²⁰ performed on rabbits inoculated probably with the same organism as in our experiments The inoculations were made during a period of starvation, and on the second day after inoculation the output of nitrogen exceeded the normal by 28.4 to 51.9 per cent Therise in nitrogen elimination is quite comparable to that observed in our experiments below except that here the animals were not starving and the fever appeared several days later These figures probably represent in a general way the increase in nitrogenous metabolism which may be observed in febrile conditions in man The particular advantage of such experiments, as those of May on animals, is the fact that one has a fairly adequate control period which cannot be so readily obtained in similar diseases in man

One other phase of the question, which is of the utmost practical importance in the treatment of fever in man, especially typhoid fever as shown by Shaffer and Coleman, is the diet The lower specific dynamic action of carbohydrate in comparison to protein and the protective action of carbohydrate on protein is well known May called attention, in his experiments in 1894, to the protective action of carbohydrate on body protein By the use of diets high

¹⁸ Linser and Schmid *Deutsch Arch f Klin Med*, lxxix, p 514, 1904

¹⁹ Krehl *Clinical Pathology*, Amer Edit by Hewlett, 1907, p 406

²⁰ May *Zeitschr f Biol*, xxx, p 1, 1894

in caloric value and rich in carbohydrate, Shaffer and Coleman²¹ have shown it possible to retard and even prevent the febrile loss of body protein nitrogen in subjects of typhoid fever. This is of particular interest in this connection in view of the inhibitory influence of carbohydrate upon the excretion of creatine by starving animals.

EXPERIMENTAL PART

Nine experiments are reported on rabbits inoculated with *Bacillus surpesticus*²² and three experiments upon animals whose temperature was raised artificially. The routine procedures in the first series of experiments consisted in determinations of total nitrogen, urea, ammonia, creatinine, creatine, chlorides, phosphates, potassium, and the routine clinical examination of the urine, together with morning and evening temperature observations during the fever period, and a previous control period of four or more days. In the last three experiments determinations of total nitrogen, creatinine, creatine and chlorides were made.

The analytical methods employed were Kjeldahl method for total nitrogen, Benedict method for urea,²³ Folin methods for ammonia and creatinine,²⁴ Benedict-Myers modification of the Folin method for creatine,²⁵ Volhard-Harvey²⁶ method for chlorides, titration with uranium nitrate for phosphates and Drushel's method for potassium.²⁷

The rabbits were all healthy animals, females being used because it is somewhat easier to compress the bladder. Prior to beginning the experiment, the animals were placed upon a uniform carrot diet for a period of several days. During the experiment the bladder was squeezed out at a definite time each morning, the animal weighed, and then given its daily ration which was ordinarily consumed during the day in the control period. After inoculation or placing in an incubator the appetite of the animals declined, obvi-

¹ *Loc cit*, also Coleman *Amer Journ of Med Sci*, cxliii, p 77, 1912

² A preliminary report of these experiments was made at the 1911 Meeting of the Society of Biological Chemists, cf *Proceedings this Journal*, vi, p 11, 1912

³ Benedict *this Journal*, viii, p 405, 1910

⁴ Folin *Amer Journ of Physiol*, viii, p 45, 1905

⁵ Benedict and Myers *ibid*, viii, p 397, 1907

²⁶ Harvey *Arch of Int Med*, vi, p 12, 1910

⁷ Drushel *Amer Journ of Sci*, lxxvi, p 555, 1908, also Myers *this Journal*, vi, p 122, 1909

ously introducing an important factor into the figures for the nitrogen elimination. However, starving animals would have been less satisfactory, for several reasons, the most important of which is the elimination of creatine which develops very quickly in the rabbit during inanition.

The 350-400 grams of carrots (contain 0.6-0.7 gram N) would appear to furnish a fairly adequate dietary for the rabbit, generally close to 100 calories per kilogram, with 10 per cent in the form of protein. The rabbit with its large skin area should require a comparatively high caloric intake, but this would appear to have been well supplied. Owing to the exhaustion of the old carrot supply (July 1911), rabbits O, P and Q were fed 300 grams of new roots and 200 grams of the green "tops," thus making the 500 grams recorded in the tables. No detailed protocols, aside from the tabulated results of the urine analyses, appear to be necessary.

The first series of experiments was carried out at the Bender Laboratory at the suggestion of Dr. Ordway with the idea that some correlation might be observed between our chemical findings and the histological data obtained in independent experiments on the same animals by Ordway, Kellert and Husted.³ We were able to detect little relationship between the lesions observed in the various organs and the composition of the urine. Our chemical data were found to be chiefly of interest from the standpoint of creatinine, and on this account figures for the potassium, chlorides, phosphates and likewise ammonia have not been included.

Below is given, however, a brief summary of the results obtained for the elimination of these constituents. Data on the excretion of potassium were obtained in the first seven experiments. The excretion appeared to follow closely in all cases the food intake, although this was rather high to have allowed the demonstration of changes due to fever or accompanying conditions. In general, with the development of fever, there was an increase in the chloride elimination, followed by a very decided decrease not accounted for by the decreased intake. This is believed to have been due to a decreased kidney (tubular) permeability. The phosphate elimination showed an increase during the fever period in certain of the experiments, while in others there was little change.

The figures for ammonia are also not recorded, for the reason that with one possible exception, Rabbit M, no indication was observed of an increased excretion of ammonia. Normally, the rabbit eliminates practically no ammonia, as might be expected from the nature of its dietary. Though the reaction of the urine was frequently observed to be acid at the height of the fever, sufficient time hardly elapsed to have caused a very great depletion of the supply of basic elements in the body.

³ Ordway, Kellert and Husted *Journ of Med Res*, LVIII, p. 41, 1913

TABLE I *Rabbit A*

DATE 1911	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL OF URINE	SP GR	CREAT- ININE N	CREA- TINE N
			p m	a m				
February and March	kgms	grams	C	C	cc		mgms	mgms
20	1 40	350	38 3	36 7	235	1 018	18 4	0
21	1 39	350	39 5	37 8	250		12 3	0
22	1 43	350	37 8	36 1	275	1 012	19 0	0
23	1 36	350	37 2	38 1	250	1 014	16 1	0
24	1 40	350	38 7	38 6	240	1 016	15 3	0
Average 15-24					247	1 015	17 2	
25	1 40	350	37 8	37 8	240	1 021	22 6	0
26	1 36	350	38 5	38 3	260	1 020	19 6	0
27	1 36	350		40 1	250		18 1	0
28	1 34	200		40 1	200	1 014	21 5	0
1	1 24	180	40 6	40 3	75	1 024	22 2	0
2	1 28	70	40 1	40 0	60	1 028	20 7	0
3	1 29	160	39 8	38 6	120	1 020	18 4	0
4	1 32	280	39 2	38 6	175	1 025	16 7	2 1
5	1 26	300	40 8	38 7	235	1 014	18 2	2 1
6	1 24	300	38 3	40 0	200	1 020	16 4	0
7	1 25	300	39 5	39 3	200	1 016	17 4	0 5

TABLE II *Rabbit C*

February and March	kgms	grams	C	C	cc		mgms	mgms
20	1 75	350	37 5		218	1 017	25 8	
21	1 76	350	37 5	37 8	275	1 019	25 8	
22	1 72	350	37 8	37 8	275	1 019	25 8	
23	1 77	350	38 3	38 8	260	1 020	18 4	
24	1 75	350	39 1	38 4	220	1 022	28 2	
Average 15-24					246	1 018	25 4	
25	1 65	350	38 6		300	1 016	32 6	0
26	1 66	350	38 5		250	1 020	25 4	0
27	1 67	350	38 2		350		33 2	0
28	1 70	350	39 6		275	1 018	22 3	0
1	1 65	300	40 6	38 6	235	1 012	37 5	0
2	1 52	280	39 9	40 5	50	1 030	21 8	0
3	1 47	90	41 0	40 3	60	1 035	39 4	0
4	1 47	80	40 6	38 3	50	1 036	29 1	5 6

Rabbit A inoculated subcutaneously on February 24 with 0.5 cc of a 24-hour bouillon culture of *Bacillus supepticus*. Pulse on February 21, 180, on February 28, 280. Animal recovered.

Rabbit C inoculated subcutaneously on February 24 with 0.5 cc of a 24-hour bouillon culture of *Bacillus supepticus*.

TABLE III
Rabbit H

DATE 1911	BODY WEIGHT kgms	CARROT DIET grams	TEMPERATURE		VOL OF URINE cc	SP GR	TOTAL N grams	UREA N grams	URLA N per cent	CREAT- ININE N mgms	CREAT- ININE N per cent	CREATINE N mgms
			p m	a m								
June				°C								
1	1 70	350	39 8	39 5	260	1 018	0 70	0 45	60	25 1	3 6	0
2	1 71	350	39 5	39 2	250	1 013	0 55	0 33	60	25 1	4 6	0
3	1 69	350	40 2	40 1	260	1 010	0 50	0 30	60	25 8	5 1	0
4	1 70	350	39 7	39 7	230	1 015	0 42	0 32	76	25 8	6 0	0
Aver												
1-4					250	1 013	0 53	0 35	66	25 5	5 0	0
5	1 67	343	40 9	40 3	230	1 015	0 63	0 48	76	31 7	5 0	0
6	1 68	317	41 0	41 4	250	1 018	0 72	0 61	84	31 7	4 4	0
7	1 64	200	42 0	41 8	152	1 017	0 69	0 62	90	34 7	5 0	0
8	1 52	0	41 7	41 1	70	1 015	0 83	0 70	84	35 4	4 2	0
9	1 47	H O, 16 cc	40 0	39 1	33	1 015	0 25	0 24	96	17 2	6 9	5 4

Rabbit H inoculated subcutaneously on June 4 at 12 30 p m with 0 5 cc of 24-hour bouillon culture of *Bacillus surpescus*. Urine strongly acid on last two days, and on last day contained albumin, a few pus cells and many hyaline casts. Rabbit died at 10 a m on June 9.

TABLE IV
Rabbit I

DATE 1911	BODY WEIGHT	CARHOT DIET	TEMPERATURE		VOL. OF URINE	SP GR	TOTAL N	UREA N	CREAT- ININF N	CREAT- ININE N	CREATINE N
			p m	a m							
June	grams	grams	C	C	cc		grams	per cent	mgms	per cent	mgms
1	2 02	350	39 2	38 9	230	1 016	0 92	0 64	27 8	3 0	0
2	1 99	350	39 2	39 0	275	1 017	0 78	0 66	27 8	3 5	0
3	2 00	350	39 5	39 2	230	1 017	0 91	0 70	25 8	2 8	0
4	1 99	350	39 1	39 1	265	1 017	0 79	0 68	26 1	3 3	0
Aver 1-4					250	1 017	0 85	0 67	26 9	3 1	
5	1 96	350	39 8	39 9	237	1 017	0 94	0 73	39 1	3 2	4 0
6	2 05	350	39 5	40 0	185	1 020	0 56	0 42	25 8	4 6	3 3
7	2 05	350	39 8	40 1	265	1 015	0 70	0 57	29 1	4 1	0
8	1 83	0	41 7	40 7	300	1 013	1 04	0 77	34 7	3 3	0
9	1 70	H ₂ O, 24 cc	40 3	37 6	96	1 012	0 88	0 63	30 1	3 4	0

Rabbit I inoculated subcutaneously on June 4 at 12 30 p m with 0.5 cc of a 24-hour bouillon culture of *Bacillus suspensus*. Urine faintly acid on last day, and on the last two days contained albumin. Animal died at 2 p m on June 9 with convulsions, was found to be pregnant.

TABLE V
Rabbit K

DATE 1911	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL OF URINE	SP GR	TOTAL N	URIA N	CREAT- ININE N		CREAT- ININE N	CREATINE N
			p m	a m					mgms	per cent		
June and July	kgms	grams	°C	C	cc		grams	grams	mgms	per cent	mgms	
	1 84	400	40 2	40 0	315	1 017	1 05	0 85	28 8	2 7	10 3	
	1 84	400	40 0	39 9	315	1 015	0 75	0 70	32 4	4 3	2 1	
	1 84	400	40 0	40 0	320	1 015	0 94	0 85	32 4	3 4	7 4	
	1 85	400		40 0	282	1 017	0 84	0 74	30 5	3 6	10 0	
	1 84	400	39 8	39 8	310	1 015	0 85	0 73	28 9	3 4	6 8	
Average 22-26												
					308	1 016	0 89	0 78	30 6	3 4	6 9	
27	1 84	400	40 2	40 2	300	1 015	0 94	0 78	31 9	3 3	0	
28	1 86	400	41 0	39 8	285	1 015	1 03	0 89	30 1	3 0	0	
29	1 85	400	40 0	40 8	295	1 014	0 86	0 75	30 5	3 5	1 9	
30	1 84	400	40 7	41 4	275	1 015	0 87	0 74	30 8	3 5	2 6	
1	1 66	150 H ₂ O, 25 cc	42 7	39 9	153	1 019	1 33	1 09	38 3	3 0	4 6	

Rabbit K inoculated in the usual way at 5.30 p.m. on June 26, pregnant and aborted on July 1, died at 9.45 a.m. on July 2. The fact that creatinine was excreted during the control period finds probable explanation in the fact that the animal was pregnant. This might also be a possible explanation of the excretion of creatinine by the previous animal after inoculation, but previous to the onset of the fever.¹¹

¹¹ Cf. Mellanby *Proc Roy Soc., B*, lxxxvi, p. 88, 1913.

TABLE VI
Rabbit M

DATE 1911	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL OF URINE	SP GR	TOTAL N	UREA N	UREA N	CREAT- ININE N	CREAT- ININE N	CREATINE N
			p m	C								
	kgms	grams		C	cc		grams	per cent	mgms	per cent	mgms	mgms
June and July												
22	1 85	400	39 7	39 6	315	1 015	0 51	0 49	25 4	5 0	0	0
23	1 82	400	39 7	39 7	280	1 015	0 55	0 46	24 2	4 4	0	0
24	1 85	400	40 0	39 6	298	1 015	0 52	0 44	24 0	4 6	0	0
25	1 84	400	39 5		295	1 015	0 57	0 45	24 5	4 3	0	0
26	1 82	400	39 6	39 5	320	1 015	0 53	0 45	24 8	4 7	0	0
Average 22-26					302	1 015	0 53	0 46	24 6	4 6	0	0
27	1 82	400	40 0	40 1	307	1 017	0 50	0 44	26 7	5 3	0	0
28	1 80	390	40 0	40 0	253	1 014	0 64	0 52	26 3	4 1	0	0
29	1 84	380	40 9	41 5	307	1 014	0 60	0 49	29 7	4 9	0	0
30	1 79	0	42 0	40 8	167	1 013	0 91	0 79	32 4	3 5	0	0
1	1 61	0	39 5		25	acid		0 34	5 6		2	2

Rabbit M inoculated in the usual way at 5 30 p m on June 26, small amount of albumin detected in the urine on June 29, urine on morning of last two days observed to be acid, rabbit died in convulsions at 3 p m on June 30

TABLE VII
Rabbit O

DATE 1911	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL OF URINE	SP OR	TOTAL N	UREA N		CREAT- ININE N	CREAT- ININE N	
			p m	a m				grams	per cent		mgms	per cent
July		grams	°C	°C	cc.							
16	1 95	500	40 0	39 5	365	1 019	0 87	0 66	76		26 8	3 1
17	1 94	500	40 0	39 8	335	1 024	1 13	0 88	78		31 0	2 7
18	1 95	500	39 8	39 3	300	1 021	0 83	0 62	74		31 0	3 7
19	1 95	500	39 6	39 5	367	1 018	0 86	0 69	80		31 3	3 7
20	1 95	500	39 9	39 6	330	1 022	0 97	0 68	70		30 1	3 1
Aver 9-20					328	1 021	0 85	0 64	76		29 2	3 4
21	1 95	500	40 0	40 3	330	1 021	1 05	0 82	78		28 9	2 7
22	1 95	500	39 7	39 7	306	1 022	0 96	0 74	77		30 1	3 0
23	1 97	500	40 8	40 7	208	1 021	0 90	0 68	76		31 0	3 4
24	1 95	500	41 4	41 1	320	1 019	0 96	0 66	69		36 3	3 8
25	1 92	refused	42 2	41 1	140	1 019	0 94	0 79	73		38 3	4 0
26	1 81	food but	41 5	40 9	190	1 014	1 65	1 49	90		47 9	2 9
27	1 75	drank H ₂ O	41 1	40 7	50	1 028	1 28	1 10	90		33 4	2 6

Rabbit O received 5 cc of a bouillon culture of *Bacillus subtilis* by mouth at 5 p m July 12 on an empty stomach but no infection apparently ensued At 5 30 p m on July 20, 0 2 cc of a 24-hour bouillon culture was given subcutaneously Animal died at 12 30 p m July 27

TABLE VIII
Rabbit P

DATE 1911	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL OF URINE	SP GR	TOTAL N	UREA		CREAT- ININE N	CREAT- ININE N	CREATINE N
			p m	a m				grams	per cent			
July	grams	grams	C	°C	cc		grams	grams	per cent	mgms	per cent	
9	2 56	500	39 5	39 5	305	1 021	0 08	0 57	88	35 1	5 2	0
10	2 34	500		40 0	235	1 026	0 86	0 59	69	35 1	4 1	0
11	2 37	500	41 1	39 2	305	1 020	0 75	0 61	81	35 1	4 7	0
12	2 38	500	40 0	39 6	265	1 025	0 92	0 68	74	35 1	3 8	0
average 9-12					282	1 023	0 80	0 61	76	35 1	4 4	0
13	2 36	500	40 2	40 3	300	1 023	0 85	0 65	77	35 1	4 1	0
14	2 40	500	39 7	39 2	325	1 024	0 83	0 61	74	35 1	4 2	0
15	2 36	500	39 7	40 6	306	1 019	0 59	0 44	75	27 0	4 6	0
16	2 32	300	41 9	41 9	325	1 019	1 05	0 81	77	39 7	3 8	0
17	2 22	100	41 9	41 7	107	1 020	1 07	0 82	77	40 5	3 8	0
18	2 17	0	41 3		29		0 26	0 20	77	16 7	6 4	0

Rabbit P received 0.2 cc of the usual culture subcutaneously at 5 p m July 12 and died during the night of July 17

TABLE IX

Rabbit Q

DATE 1911	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL. OF URINE	SI OR	TOTAL N	URIA N		CREAT- ININE N	CREAT- ININE N	
			p m	a m				grams	per cent		mgms	per cent
July				°C	cc							
17	1 63	500	40 3	39 8	205	1 030	0 76	0 51	67			
18	1 60	500	40 0	39 5	343	1 018	0 46	0 32	70			
19	1 59	500	40 0	40 0	342	1 019	0 65	0 50	77			
20	1 59	500	40 7	40 0	330	1 020	0 54	0 41	76			
Average 9-20					303	1 020	0 50	0 38	68	days 8-14	19 5	3 5
21	1 57	500	40 0	40 2	325	1 021	0 61	0 44	72		17 6	2 9
22	1 61	500	40 1	40 1	226	1 025	0 59	0 45	76		17 6	3 0
23	1 62	500	40 0	40 0	296	1 024	0 81	0 64	76		20 2	2 4
24	1 57	500	41 2	40 9	320	1 019	0 62	0 48	77		21 3	3 4
25	1 61	300	40 7	41 7	285	1 020	0 71	0 53	75		21 7	3 1
26	1 53	300	42 1	41 4	172	1 022	0 91	0 77	85		26 4	2 9
27	1 54	215	41 4	41 1	75	1 027	0 89	0 74	83		25 1	2 8
28	1 56	225	41 6	40 6	100	1 040	0 92	0 74	80		25 1	2 7
29	1 50	70	41 1	38 6	85	1 026	1 50	1 25	83		26 3	11 8

Rabbit Q received 5 cc of the usual bouillon culture by mouth at 5 p m July 12 on an empty stomach, but no infection followed. On July 21 the animal was given a subcutaneous injection of 0.2 cc of the culture. Rabbit died July 29.

TABLE X
*Rabbit 45*³⁰

DATE 1912	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL. OF URINE	SP OR	TOTAL N	CREAT- ININE N	CREAT- ININE N
			p m	a.m.					
July and August	kgms	grams	°C	°C	cc		grams	mgms	per cent
31	1 87	300	39 8	39 0	236	1 017	0 67	22 0	3 3
1	1 74	300	39 5	39 3	268	1 017	0 84	27 0	3 2
2	1 57	350	39 6	39 1	230	1 018	0 68	23 0	3 4
3	1 70	350	39 9	39 0	242	1 018	0 64	24 7	3 9
4	1 65	350	39 3	39 5	300	1 016	0 68	27 4	3 7
5	1 62	350	39 6	39 5	225	1 017	0 74	27 4	4 0
Average 31-5					250	1 017	0 71	24 6	3 5
6	1 64	250	40 9	41 1	85	1 023	0 41	23 8	5 8
7	1 56	120	41 0	41 7	40	1 027	0 82	30 6	3 7
8	1 41	110	41 7	43 6	30	1 030	0 99	32 1	3 2
9	1 42	240	39 0	39 1	73	1 031	1 07	28 7	2 7
10	1 52	350	39 2	39 5	183	1 018	0 72	21 2	2 9
11	1 58	350	39 3	39 6	305	1 016	0 70	20 8	3 0

Rabbit 45 was placed in the incubator on the morning of August 5 and kept there continuously for three days, except for about one hour each morning. During the incubator period the animal drank a small amount of water. The first day after being removed from the incubator the rabbit appeared sick, but was apparently normal on the following day.

³⁰ In this last series of experiments we were assisted by Mr Adolph Bernhard.

TABLE XI. Rabbit 47

DATE 1913	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL. OF URINE	SP GR.	TOTAL N	CREAT- ININE N	CREAT- ININE N
			p.m.	a.m.					
January	kgms	grams	°C	C	cc.		grams	mgms	per cent
16	1 58	320	39 4	39 0	225	1 012	0 27	20 3	7 5
17	1 59	340	39 6	39 0	235	1 012	0 33	19 6	5 9
18	1 59	325	39 5	39 0	270	1 010	0 31	16 0	6 5
19	1 56	350	39 7	38 7	268	1 013	0 44	18 4	4 2
20	1 60	350	38 7	39 0	200	1 010	0 33	19 2	5 8
Average 16-20					240	1 011	0 34	18 7	5 5
21	1 48	110	41 2	40 6	58	1 018	0 33	23 2	7 0
22	1 35	35	40 2	41 7	28	1 028	0 62	25 9	4 2
23	1 33	0	41 6	41 4	25	1 028	0 73	26 9	3 7
24	1 46	200	39 4	38 0	40	1 030	0 82	21 5	2 6
25	1 56	310	38 0	39 1	140	1 020	0 44	20 1	4 6

Rabbit 47 was placed in the incubator at 2 p.m. on January 20 and kept there continuously, except for the time necessary to take temperatures, etc., until 9 a.m. on the 23rd. On the last two of these three days the reaction of the urine was observed to be acid.

TABLE XII Rabbit 49

DATE 1913	BODY WEIGHT	CARROT DIET	TEMPERATURE		VOL. OF URINE	SP GR.	TOTAL N	CREAT- ININE N	CREAT- ININE N
			p.m.	a.m.					
January	kgms	grams	C	°C	cc		grams	mgms.	per cent
16	1 36	350	39 7	39 2	290	1 010	0 12	18 4	15 3
17	1 32	350	39 7	39 3	315	1 011	0 19	21 1	11 1
18	1 32	350	40 0	39 3	230	1 012	0 23	18 3	8 0
19	1 34	350	39 7	39 1	245	1 012	0 28	20 0	7 1
20	1 42	350	38 1	39 5	180	1 016	0 22	16 4	7 5
Average 16-20					252	1 012	0 21	18 8	9 0
21	1 39	350	42 0	39 1	220	1 010	0 35	21 2	6 1
22	1 26	80	40 7	42 1	30	1 022	0 23	18 8	8 5
23	1 29	50	41 5	41 5	35	1 032	0 72	24 1	3 3
24	1 36	350	38 9	39 0	205	1 016	0 60	21 4	3 6
25	1 45	350	39 1	39 1	100	1 027	0 19	15 0	7 9

Rabbit 49 was placed in the incubator at 2 p.m. on January 23. The temperature at 8 45 p.m. was found to be 42.1°C, and it was thought best to remove the animal to the room. The next morning (22nd) the rabbit was again placed in the incubator and kept there for the two following days.

Discussion

The results of the foregoing experiments can most advantageously be discussed together. The parallel between the body temperature and the total nitrogen, urea, and creatinine is quite striking, though in considerable measure probably due to the shortness of the fever, generally five to eight days. In all cases the highest elimination of nitrogen has been found to coincide with the highest temperature. The relation between the total nitrogen and urea, as shown by the percentage of urea nitrogen, does not markedly differ from the normal. In general, during the fever period there was a slight increase in the per cent of urea nitrogen. At the height of the fever this was sometimes accompanied by a still further increase, and in other cases by a decrease.

The relation between the body temperature and the amount of the creatinine elimination is most interesting. As soon as the body temperature rises noticeably above the normal level, there is an accompanying increase in the excretion of creatinine, and with a decline in the febrile temperature there is an immediate fall. Though, in general, the creatinine follows the increased total nitrogen excretion, there is a very noticeable lag at the height of the fever, the average figures disclosing a fall of from 3.8 per cent to 3.3 per cent in terms of total nitrogen. The maximum temperature, about 42°C, was found to be accompanied by the highest creatinine elimination, the percentage increase over the control elimination averaging 36 per cent—64 per cent in one case—as recorded in Table XIII.

Creatine was generally found to be eliminated after the crisis of the disease. This was also the case in one other experiment not reported, Rabbit L. The recent experiments of Myers and Fine³¹ on starving rabbits make it evident that the creatine of the urine, at least in that condition, has the origin generally assumed, viz, from the setting free of creatine in the absorption of muscle tissue. Though creatine may be an index as to the amount of a certain kind of abnormal catabolism, the creatine itself bears no uniform relation to the endogenous total nitrogen excretion.

To secure further light on the factors causing the increased elimination of creatinine during fever, three animals were placed in a

³¹ Myers and Fine *Proc Soc Exp Biol and Med*, v, p 12, 1912

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large incubator at about 38.5°C. As is well known, the rabbit is easily susceptible to external changes in temperature and in this way a typical hyperthermia was easily produced with maximum temperatures varying between 41.7° and 43.3°C.

As is shown in Tables X, XI and XII, the rise in temperature here was likewise found to be accompanied by an increased elimination of creatinine, the maximum output being noted on the day of the highest temperature. The increased excretion was of the same intensity as that observed in the previous series of experiments. It is of interest to note that here no creatine was found to appear in the urine, possibly indicating that there was no abnormal destruction of muscle tissue. Inasmuch as no creatine was found no tabulation was made. The maximum total nitrogen excretion in this series did not appear at the time of the highest temperature, but on the day following. The coincidence in the previous series finds probable explanation in the fact that the highest temperature and the highest total nitrogen just preceded the premortal fall.

TABLE XIII

EXPERIMENT	AVERAGE DAILY TOTAL N CONTROL PERIOD	HIGHEST TOTAL N FEVER PERIOD	AVERAGE DAILY CREATININE N CONTROL PERIOD	HIGHEST CREATININE N FEVER PERIOD	INCREASED CREATININE EXCRETION DUE TO FEVER
	grams	grams	mgms	mgms	per cent
A			17.2	22.2	29
C			25.4	39.4	55
H	0.53	0.83	25.5	35.4	39
I	0.85	1.04	26.9	34.7	29
K	0.89	1.33	30.6	38.3	25
M	0.53	0.91	24.6	32.4	32
O	0.85	1.65	29.2	47.9	64
P	0.80	1.07	35.1	40.5	15
Q	0.56	1.50	19.5	26.4	35
Average increased excretion of creatinine					36
45	0.71	1.07	24.6	32.1	31
47	0.34	0.82	18.7	26.9	44
49	0.21	0.72	18.8	24.1	28
Average increased excretion of creatinine					34

In the above table are summarized the more important data in connection with the excretion of creatinine. Percentage figures for the increased excretion of total nitrogen during the height of the fever are not given owing to the fact that it appeared impossible to compute reliable figures. During the control period a number of the animals showed a plus nitrogen balance, with nitrogen intakes varying between 0.6 and 0.7 gram. Furthermore, during the fever period, they were burning increased amounts of their own tissue due in part to the decreased food intake. However, it appears that there was a greater increase in the excretion of nitrogen during pyrexia in the first series of experiments than in the second.

CONCLUSIONS

The excretion of creatinine closely follows the rise in temperature during fever, whether the hyperthermia is of infective origin or artificially induced. The highest continued temperature (about 42°C) has been found to be accompanied by the highest creatinine elimination.

In nine experiments on rabbits inoculated with *Bacillus surpeticus*, the percentage increase at the height of the fever over the control elimination averaged 36 per cent. In three experiments, where hyperthermia was artificially induced, the average increase was 34 per cent. This is believed to show that the increased elimination of creatinine is due entirely to the hyperthermia.

The view is expressed that the increased creatinine elimination during fever still represents the normal endogenous metabolism, which is proceeding here at an abnormal intensity due to the high temperature. It is possible that this is in accord with the law of the increased velocity of chemical reactions at increased temperatures.

It is suggested that the amount of the increased creatinine excretion may be of value in indicating the increase in nitrogenous metabolism due to simple pyrexia. The increased excretion of nitrogen in physiological fever corresponds very well with the increased excretion of creatinine (35 per cent), but in toxic fevers the excretion of nitrogen may be much greater.

In the series of toxic fevers, creatine was generally found to be excreted, and when present was observed, as a rule, following the

crisis of the fever In the series of physiological fevers an elimination of creatine was not detected It is reasonable to believe that creatine is excreted during fever, because the protein is drawn upon to supply unusual demands (*e g* , to supply energy in the absence of carbohydrate), and in this abnormal catabolism, creatine is set free more rapidly than the body can oxidize it

In fever, creatinine, though increased, still appears to indicate the amount of a certain type of normal endogenous metabolism, while creatine possibly indicates the amount of abnormal endogenous metabolism

THE NORMAL PROTEIN METABOLISM OF THE RAT

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The highly interesting results obtained by Mendel and Osborne¹ on feeding single specific proteins to rats lend added interest to any detailed information that may be obtained concerning the nature of the metabolism of the rat as compared with that of any other animal or of man. The small size of the animal has heretofore prevented the accumulation of much information of a quantitative nature. The total urinary nitrogen determinations of Mendel and Osborne and those of Hatai² represent so far as we know all that has been done in this line. By means of the new microchemical method,³ recently published from this laboratory it is possible to obtain more detailed figures. Because of the limited quantity of urine comprising the twenty-four-hour amounts, some changes in these methods were found necessary. In every case the twenty-four-hour urine of each animal (5-50 cc in amount) was made up to 50 cc. From this stock solution portions were taken directly for ammonia, uric acid, creatinine and creatine determinations. For total nitrogen and urea determinations a dilution of a part of this solution to twice its volume brought the nitrogen content to the desired amount ($1 \pm$ mgm per 1 cc). In the smallest rats this second dilution was unnecessary, the quantity being sufficiently reduced in the first dilution.

The uric acid analysis followed the general scheme of its determinations in human urine as published by Folin and Denis.⁴ Ten cubic centimeters of rat urine were found to contain a sufficient quantity of uric acid for the determination. A drop of acetic acid

¹ Carnegie Institution of Washington, publication 156, Pts I and II

² *Amer Journ of Physiol*, xiv, p 102, 1905

³ *This Journal*, xi, 1912

⁴ *Ibid*, xiv, p 95, 1913

(instead of hydrochloric) was used to acidify when driving off the hydrogen sulphide. After the reaction with the uric acid reagent and sodium carbonate the blue solution was filtered into a 25 cc volumetric flask and made up to the mark with washings. It was then read against a standard of 1 mgm of uric acid in 100 cc.

The creatinine determinations offered at the beginning considerable difficulty owing to the small amount of creatinine and its great dilution in rat urine. The original standard, potassium bichromate solution, could not be used and had to be replaced by creatinine solutions of known concentration. Other factors had to be modified—the amounts of picric acid and alkali, and the time necessary for the development of the maximum color had to be worked out. Our standard creatinine solutions were made of approximately the same concentration as the urine. These solutions and the urine were then treated exactly alike and, by developing the color simultaneously in both, constant and seemingly reliable results were obtained. The modified procedure is as follows:

To 5 cc of urine and to 5 cc of the standard creatinine solution, each in a 25 cc volumetric flask, add 2.5 cc of saturated picric acid solution from a burette, and then 1 cc of 10 per cent sodium hydroxide solution. Let stand for ten minutes, fill up to the mark with water and then determine the color as in the original method.

The creatine determination was made, in terms of creatinine, by the same method and appeared as an addition to the previously obtained creatinine figure. It was converted into creatinine by adding 0.5 cc of 2N HCl to 5 cc of urine in a 100 cc Erlenmeyer flask, evaporating to rather less than 2 cc volume on a water bath, and continued heating at this volume for two hours. To maintain the volume a small top-shaped glass bulb was inserted in the mouth of the flask for condensation. At the end of the time indicated the volume was again made up to approximately 5 cc by adding water to the flask on a balance until the weight was the sum of 5 grams and the previously-taken weight of the flask. Beyond this the treatment was exactly the same as with creatinine except that an additional 0.5 cc of NaOH was added to neutralize the HCl used.

Each rat was kept in a specially constructed small cage resting on top of a funnel. Each day's urine was collected in 2 cc of normal hydrochloric acid. The twenty-four-hour aspect is only approximate since no attempt was made to have the bladder emptied at the end of each day. The duration of the experiment is long enough, however, so that the average of all the figures should come close to the true twenty-four-hour results. The urines were analyzed in the case of rats M, X and A over a period of nine days, and in the case of rat G, fifteen days.

In tables 1 and 2 are given the analyses of a series of approximately twenty-four-hour urines obtained from two large mature rats, a female (M) weighing 290 grams and a male (X) weighing 197 grams. To save space only the last six days are recorded.

Table 3 represents a summary of the results recorded in tables 1 and 2, together with summaries of the results obtained from two young growing rats, A and G, weighing 40 to 50 grams. We have made several other series of similar studies but these deal chiefly with tumor-bearing rats and will be described elsewhere by Ordway and Morris.

The rats were kept on a purine-free diet consisting of powdered crackers and water.

It will be seen, from examination of the average results, that the percentage composition of rat urine differs but little from that of human urine. Being small animals but voracious feeders the total nitrogen per kilo of body weight is much larger than in the case of man. The percentage relationship between the amounts of total nitrogen, urea nitrogen and ammonia nitrogen is almost the same as in man. Creatinine nitrogen shows a somewhat larger value for the rats, reaching 15 mgm per kilo in the mature rats, while man usually eliminates 7-11 mgm. Small amounts of creatine were always found in the urine. This is rather interesting in view of the excessive feeding of rats and the suggestion of Folin and Denis⁵ that the creatine in the urine of children might be due to an "excessively high level of protein consumption."

The most striking and interesting feature of the analyses is the fact that the urine of rats contains quite as much uric acid in proportion to body weight as does human urine. Through the

⁵ This *Journal*, XI, p 253, 1912

TABLE 1
Female rat weighing 290 grams (M)

DATE (DEC.)	16		18		19		20		21		AV 24 HOURS	
	mgm	per cent	mgm	per cent	mgm	per cent	mgm	per cent	mgm	per cent	mgm	per cent
Total N	172.5	100.0	184.5	100.0	165.0	100.0	190.5	100.0	155.0	100.0	173.5	100.0
Urea N	147.9	86.0	139.1	75.4	130.0	78.8	150.0	78.7	103.8	67.0	143.2	77.3
Ammonia N	9.1	5.3	9.4	5.1	9.5	5.8	9.4	4.9	8.2	5.3	9.1	5.2
Uric Acid N	0.52	0.3	0.73	0.4	0.71	0.43	0.83	0.43	0.65	0.42	0.69	0.40
Creatinine N	4.8	2.8	4.7	2.6	3.9	2.4	4.3	2.2	4.7	3.0	4.5	2.65
Creatinine + Creatine N	5.0	2.9	4.6	2.6	3.6	2.3	4.7	2.5	4.7	3.0	4.7	2.71

TABLE 2
Male rat weighing 197 grams (X)

DATE (DEC.)	16		17		18		19		20		21		AV 24 HOURS	
	mgm	per cent	mgm	per cent	mgm	per cent	mgm	per cent	mgm	per cent	mgm	per cent	mgm	per cent
Total N	144.0	100.0	127.0	100.0	119.0	100.0	118.0	100.0	116.0	100.0	132.0	100.0	126.0	100.0
Urea N	115.3	80.1	109.8	86.5	102.7	86.3	88.7	75.2	101.0	87.1	118.1	89.5	105.9	84.0
Ammonia N	8.2	5.7	7.1	5.6	6.6	5.5	6.3	5.4	5.4	4.7	6.9	5.2	6.7	5.3
Uric Acid N	0.43	0.3	0.75	0.59	0.62	0.52	0.43	0.36	0.62	0.54	0.52	0.41	0.52	0.41
Creatinine N	3.1	2.1	3.3	2.6	2.6	2.2	3.0	2.5	2.4	2.1	3.2	2.4	2.9	2.30
Creatinine + Creatine N	3.2	2.2	3.7	2.9	2.9	2.5	2.7	2.2	2.4	2.1	3.3	2.5	3.0	2.38

TABLE 3
Summary of percentages

	RAT											
	M			X			A			G		
	Max	Min	Av	Max	Min	Av	Max	Min	Av	Max	Min	Av
Weight	292 0	289 0	290 5	197 0	197 0	197 0	42 7	41 0	41 9	51 5	46 5	49 0
Urea N	86 0	67 0	77 3	89 5	75 2	84 0	78 2	64 1	71 3	87 7	61 1	76 3
Ammonia N	8 6	4 9	5 2	8 5	5 2	5 3	11 7	6 1	9 0	10 5	4 8	7 0
Uric Acid N	0 43	0 29	0 40	0 59	0 30	0 41	0 47	0 21	0 35	0 67	0 15	0 48
Creatinine N	3 0	2 2	2 65	2 6	2 1	2 30	1 3	1 0	1 08	1 7	1 1	1 37
Creatinine N + Creatine N	3 1	2 3	2 71	2 9	2 1	2 38	1 5	1 1	1 16	1 8	1 0	1 44

Summary in grams per kilo body weight *

	RAT					
	M		X		A	
	Max	Min	Max	Min	Max	Min
Total N	0 598	0 537	0 639	0 594	1 068	0 594
Urea N	0 463	0 337	0 537	0 453	0 761	0 453
Ammonia N	0 031	0 034	0 034	0 041	0 096	0 041
Uric Acid N	0 0024	0 0026	0 0026	0 0029	0 0037	0 0029
Creatinine N	0 0155	0 0147	0 0147	0 0082	0 0115	0 0082
Creatinine N + Creatine N	0 0162	0 0148	0 0148	0 0086	0 0124	0 0086

* (Figures based on average weights both of animals and metabolism products)

investigations of Wiechowski⁶ and more recently of Hunter and Givens⁷ we have learned that mammals other than man convert the greater part of the uric acid into allantoin, and the urines of such animals therefore contain very little if any uric acid. The metabolism of rats is, however, in this respect like that of man.

In view of this highly curious similarity it seemed necessary also to determine whether the blood of rats is as rich in uric acid as is human blood. Folin and Denis⁸ have recently shown that domestic animals, whose urine contains allantoin instead of uric acid, uniformly show mere traces of uric acid in the blood. The blood from six full-grown rats was collected over a little powdered potassium oxalate, and the uric acid, total non-protein nitrogen and urea were determined by the methods of Folin and Denis. The figures obtained for 100 grams of blood were as follows: Uric acid, 2 mgm, non-protein nitrogen, 38, urea, 22. The experiment was repeated twice, each time using for the analysis the mixed blood of six normal white rats, and 24 mgm and 25 mgm respectively of uric acid per 100 grams of blood were found. These are substantially the same figures as Folin and Denis found for normal human blood. The purine metabolism of rats is, therefore, like that of man and unlike that of other mammals hitherto investigated. Jones and Rohd⁹ published some experiments on purine ferments of the rat a few years ago, and one of the conclusions reached is interesting in connection with our results. "The results of this work show that the organ extracts of the rat jointly and severally are incapable of exhibiting either adenase or xantho-oxidase. There is, therefore, no way for uric acid to be formed by the purine ferments in extracts of the organs of the animal. Nevertheless rats' urine contains uric acid. From 50 cc of urine we were able to isolate enough uric acid for complete identification. Presumably the organs also contain uric acid which might be detected by methods of sufficient refinement but the substance cannot be produced by the action of organ extracts on purine bases."

⁶ *Biochem Zeitschr*, xxv, p 433, 1910

⁷ *This Journal*, xii, p 372, 1912

⁸ *Ibid*, xiv, p 31, 1913

⁹ *Ibid*, vii, p 237, 1909

It seems rather remarkable that the one animal which (excepting man) produces the most uric acid in the course of normal metabolism should lack the ferments capable of producing it. In this connection we can state that investigations conducted by J B Sumner in this laboratory have shown that aqueous extracts from rat livers are as capable of destroying uric acid as similar extracts obtained from the livers of cats and sheep. The significance of "purine ferments" as obtained from organ extracts in relation to the formation and elimination of uric acid in the course of normal metabolism is, therefore, far from clear.

IS NARCOSIS DUE TO ASPHYXIATION?

BY JACQUES LOEB AND HARDOLPH WASTENEYS

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(Received for publication, April 30, 1913)

1 The idea that the phenomenon of narcosis might be caused by an interference with oxidation is an old one. Recently it has been advocated by Verworn who tried to support it by indirect evidence¹. Yet it is obvious that if we wish to ascertain whether or not narcosis is due to a diminution in the rate of oxidations (or, as Verworn expresses it, to an asphyxiation) there is only one way to decide the question, namely by comparing the rate of oxidations in narcotized organisms with that in non-narcotized.

If we wish to carry out such experiments we must keep in mind that organisms which are capable of muscular action cannot well be used for this purpose since we know that muscular activity can easily raise the rate of oxidation in the animal body one hundred per cent. Since the narcotics cause muscular action to cease, a narcotized animal will consume less oxygen than a free-moving animal, but it would be a mistake to state that this diminution in the consumption of oxygen was the cause of narcosis. It is the effect and not the cause of narcosis. If we wish to test the asphyxiation hypothesis of narcosis we must use organisms which possess no muscles and in which therefore this source of error does not interfere with the result. The best material for this purpose is offered in the fertilized eggs. Loeb had shown in 1895 that the eggs of the sea urchin and fish cannot develop if the oxygen is withdrawn² and this result has since been extended to a number of eggs of different forms. The same author showed that the addition of a small amount of KCN also inhibits segmentation. We shall see later that KCN only has this effect if it depresses the rate of oxidations in the egg below a certain level. The suppression of

¹ Verworn *The Harvey Lectures*, 1911-12, p. 52

² Loeb *Pflüger's Archiv*, lxx, p. 249, 1895

segmentation by lack of oxygen as well as by KCN is a reversible process

It is also well known that anaesthetics inhibit the segmentation of the fertilized sea urchin egg reversibly. It was, therefore, an easy task to compare the rate of oxidations in equal quantities of fertilized eggs with and without the presence of narcotics. Warburg had already observed that phenyl urethane in the concentration in which it suppressed nuclear and cell division did not diminish the rate of oxidations. Only if an excessive amount of phenyl urethane was added, was a diminution in the rate of oxidations noticeable, but, as he correctly adds, the question is not whether it is at all possible to lower the rate of oxidations by adding narcotics but whether that concentration which suffices for narcosis lowers the rate of oxidations.³

We undertook a series of experiments on the effect of various narcotics upon the rate of oxidations in the newly fertilized eggs of *Strongylocentrotus purpuratus*. Since the method of procedure and the degree of reliability of our method have been described in previous papers no recapitulation of these points is needed.

2 Experiments with KCN We determined the minimum amount of KCN necessary to suppress cell division permanently in the newly fertilized eggs of *S. purpuratus* and found that the addition of 0.7 cc of 0.01 per cent KCN to 50 cc of sea water was the minimum amount required. We then determined the influence of various amounts of KCN upon the rate of oxidations. Newly fertilized eggs were divided into equal lots and the amount of oxygen consumed in one hour was determined. Temperature 14°C

TABLE I

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	mgm	
1 Normal sea water	0.51	1.00
2 50 cc sea water + 0.7 cc 0.01 per cent KCN	0.17	0.33
3 50 cc sea water + 0.9 cc 0.01 per cent KCN	0.15	0.29

In solution 1, the eggs segmented, in solutions 2 and 3 no segmentation occurred. The eggs were transferred from solutions 2 and

³ O Warburg *Zeitschr f physiol Chem*, lxxvi, p 305, 1910

3 to normal sea water and all developed into normal larvae. The amount of KCN necessary to suppress cell division lowers the rate of oxidations to one-third of the normal amount. A repetition of the experiment confirmed the result.

TABLE II

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	<i>mgm</i>	
1 Normal sea water	0.65	1.00
2 50 cc sea water + 0.4 cc 0.01 per cent KCN	0.34	0.52
3 50 cc sea water + 0.7 cc 0.01 per cent KCN	0.21	0.32
4 50 cc sea water + 1.0 cc 0.01 per cent KCN	0.18	0.28

The addition of 0.4 cc of KCN only retarded the development but did not suppress it. The addition of 0.7 cc of KCN suppressed cell division, and the rate of oxidations was again exactly one-third of the normal.

Similar results had previously been obtained by us for the eggs of *Arbacia*.⁴

We can, therefore, state that if the rate of oxidations of the egg is lowered to one-third of the normal amount found at 14°C, the eggs cease to segment. If the prevention of segmentation by narcotics were due to the same influence we should have to expect also a lowering of the rate of oxidations to one-third of the normal rate found at 14°C.

3 We tried a number of narcotics, various alcohols, chloral hydrate, chloroform and ethyl urethane. The minimum amount required to suppress cell division permanently was ascertained for each of these narcotics. The minimal dose varies slightly for the eggs of various individuals.

a Chloral hydrate The minimum amount required to suppress cell division is 4.2 cc of 0.5 per cent solution (made up in $\frac{M}{2}$ NaCl + KCl + CaCl₂) to 45.8 cc of sea water. Temperature 15°C.

⁴ Loeb and Wasteneys *Biochem Zeitschr*, xxxvi, p 355, 1911

TABLE III

	OXYOEN CONSUMED	RATE OF OXIDATIONS
	<i>mgm</i>	
Normal sea water	0 65	1 00
2 4 cc 0 5 per cent chloral hydrate in 50 cc sea water	0 60	0 92
4 2 cc 0 5 per cent chloral hydrate in 50 cc sea water	0 57	0 88
6 0 cc 0 5 per cent chloral hydrate in 50 cc sea water	0 57	0 88

In the solution with 4 2 cc and 6 0 cc of chloral hydrate the eggs were no longer able to segment, but they segmented and developed promptly when transferred to normal sea water. In the solution with 2 4 cc of chloral hydrate the eggs segmented. The effect of the chloral hydrate upon oxidations was practically nil. The experiment was repeated.

TABLE IV

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	<i>mgm</i>	
Normal sea water	0 51	1 00
4 2 cc 0 5 per cent chloral hydrate in 50 cc sea water	0 42	0 89

Although the segmentations were completely suppressed in this solution the influence of the chloral hydrate was practically negligible. We can say with certainty. The narcotic effect of chloral hydrate upon the egg is not due to asphyxiation or a diminution in the rate of oxidations.

b Ethyl urethane Three cubic centimeters of a 10 per cent solution (in $\frac{M}{2}$ NaCl + KCl + CaCl₂) in 50 cc of sea water are sufficient to suppress cell division.

TABLE V

	OXYOEN CONSUMED	RATE OF OXIDATIONS
	<i>mgm</i>	
Normal sea water	0 51	1 00
3 cc 10 per cent ethyl urethane in 50 cc sea water	0 46	0 98

With a few exceptions no eggs segmented in ethyl urethane, but all segmented when put back into normal sea water

c Chloroform Seven cubic centimeters of 0.5 per cent CHCl_3 in $\frac{M}{2}$ $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ were required to suppress cell division

TABLE VI

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	mgm	
Normal sea water	0.47	1.00
43 cc sea water + 7 cc 0.5 per cent chloroform	0.41	0.87

No segmentation occurred in the eggs in chloroform, but they segmented and developed normally when transferred to normal sea water. The narcotic effect of chloroform is produced without any considerable lowering of the rate of oxidations. The slight lowering observed is not quite but almost within the limits of error and cannot be considered.

d Propyl alcohol Minimum amount necessary to suppress cell division is 3.5 cc of 2 M propyl alcohol in 50 cc of sea water. In order to avoid lowering of concentration of salts the 2 M solution of alcohol was made up in $\frac{M}{2}$ $\text{NaCl} + \text{KCl} + \text{CaCl}_2$

TABLE VII

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	mgm	
In normal sea water	1.00	1.00
2.0 cc 2 M propyl alcohol in 50 cc sea water	0.90	0.90
3.5 cc 2 M propyl alcohol in 50 cc sea water	0.92	0.92
5.0 cc 2 M propyl alcohol in 50 cc sea water	0.93	0.93

In 2 cc of propyl alcohol a few of the eggs (less than 1 per cent) segmented. In 3.5 cc and 5 cc of propyl alcohol no egg segmented. A few underwent cytolysis, but as Warburg has shown, and as we were able to confirm, this does not alter the rate of oxidations in the fertilized egg. We therefore see that the narcotic effect of propyl alcohol is not accompanied by any lowering of the rate of oxidations in the egg.

e Various other alcohols The narcotic effect of various alcohols follows the rule that each successive alcohol of a series is two or

three times as efficient for narcosis as the previous one. The amount necessary for the suppression of segmentation in the egg of *S. purpuratus* found for the various alcohols was as follows

	RATE OF EFFICIENCY COMPARED WITH METHYL ALCOHOL
Methyl alcohol, 40 cc 10 M in 50 cc sea water	1
Ethyl alcohol, 50 cc 4 M in 50 cc sea water	2
Propyl alcohol, 35 cc 2 M in 50 cc sea water	6
Butyl alcohol, 35 cc M in 50 cc sea water	12

TABLE VIII

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	mgm	
Normal sea water	0.87	1.00
4 cc 10 M methyl alcohol in 50 cc sea water	0.83	0.95
5 cc 4 M ethyl alcohol in 50 cc sea water	0.73	0.84
7 cc $\frac{M}{2}$ butyl alcohol in 50 cc sea water	0.66	0.76

No eggs segmented in the solutions containing alcohol except in the methyl alcohol in which 25 per cent of the eggs segmented. When transferred to normal sea water all the eggs segmented. The rate of oxidations in these experiments is too high to account for the narcotic effect on the basis of asphyxiation. The experiment with butyl alcohol was repeated.

TABLE IX

	OXYGEN CONSUMED	RATE OF OXIDATIONS
	mgm	
Normal sea water	0.84	1.00
6.5 cc $\frac{M}{2}$ butyl alcohol in 50 cc sea water	0.66	0.79

4 *Theoretical remarks* The maximal lowering of the rate of oxidations found under the influence of narcotics was 20 per cent, in the majority of cases it was less than this. In the case of propyl alcohol it was less than 10 per cent, in the case of chloral hydrate it was about 10 per cent. Since the temperature coefficient for the rate of oxidations in the eggs is about 2 for 10°C,⁵ we can pro-

⁵ Loeb and Wasteneys *Biochem Zeitschr*, xxxvi, p. 345, 1911

duce a lowering of the rate of oxidations of 20 per cent by lowering the temperature two or three degrees, *e g*, putting the eggs into sea water of 12° instead of into 15° as in these experiments. The previous experiments of Loeb have shown that the eggs of *S purpuratus* segment not only at 12° but even at 3°C, when the rate of oxidations is less than one-half of that observed at 15°C. In addition, the experiments with KCN also show that this substance does not suppress cell division until the rate of oxidations is reduced to one-third of the normal value. From these facts we can state with certainty that the effect of narcotics upon the eggs of the sea urchin is not due to asphyxiation, a conclusion which Warburg reached also in his experiments with phenyl urethane.

Kisch⁶ has recently published experiments which show that the photodynamic effect due to oxidations is raised instead of being diminished if narcotics are added to the medium.

SUMMARY

It is shown that chloral hydrate, ethyl urethane, chloroform and various alcohols produce complete narcosis in the fertilized eggs of the sea urchin without practically lowering the rate of oxidations in the egg.

⁶ Kisch *Zeitschr f Biol*, 18, p 399, 1913

THE CHEMISTRY OF GLUCONEOGENESIS ¹

III THE FATE OF ISOBUTYRIC, ISOVALERIANIC AND ISOCAPROIC ACIDS IN THE DIABETIC ORGANISM, WITH CONSIDERATION OF THE INTERMEDIARY METABOLISM OF LEUCINE AND VALINE

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(Received for publication, April 30, 1913)

For the proper understanding of the processes involved in the metabolism of the different foodstuffs it is essential to know the fate of the individual products of metabolism in the body For the past few years this question has been the center of attack from different angles The difficulties associated with these researches appeared insurmountable at times, but with the latest developments in the application of organic chemistry to biological processes more and more light is being thrown on the subject of the intermediary metabolism of foodstuffs

There are a number of methods that have been devised of late for the prosecution of these researches, and while it must be admitted that every one of them may be associated with a certain amount of error, they have nevertheless all helped to bring to light certain definite reactions The entire subject has been recently reviewed² and much space need not be given here to this phase of the problem

In these researches animals were made diabetic by daily injections of 1 gram of phlorhizin ground up in a mortar with 10 cc of olive oil The urine was collected by catheter and at the end of each period of twelve hours the bladder was washed three times with warm distilled water

¹ Aided by a grant from the Rockefeller Institute for Medical Research

² *Uakin Oxidations and Reductions in the Animal Body*, Longmans, Green and Company, London, 1912

The urine was analyzed for its optical activity, nitrogen, glucose, ammonia, acetone, aceto-acetic acid and β -hydroxybutyric acid. In these determinations we were assisted by Mr C A Hornberger.

The original purpose of these researches was to find the chemical configuration that a substance required in order to become converted into glucose in the diabetic organism. It soon became evident that the study of this problem was so closely interwoven with questions of the intermediary metabolism of foodstuffs and substances chemically related to them, that it was deemed necessary to extend the scope of this investigation to a broader field.

The method employed in these researches permits of the study of the following problems:

I The fate of different substances in the animal body with reference to their ability to form glucose

II The fate of different substances with reference to their ability to give rise to β -hydroxybutyric acid, aceto-acetic acid and acetone

III The chemical changes that different substances undergo in the process of catabolism in the animal body before their conversion into glucose or acetone bodies

IV The influence of different substances on the metabolic processes of the diabetic organism—especially antiketogenesis

V The rôle of different substances in the pathology of diabetes

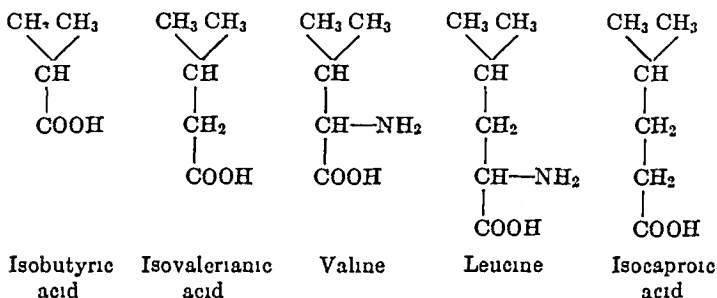
In the first two papers of this series it was shown that the normal fatty acids in the processes of catabolism in the diabetic animal undergo very definite reactions, by becoming oxidized in the β -position, giving rise to an acid with two carbons less. This conclusion was reached after having established the fact that propionic acid (when fed to diabetic dogs) is completely converted into glucose, and that of the higher fatty acids only those give rise to glucose which stand in relationship to propionic acid by having two (or a multiple thereof) carbons more. Thus *n*-valerianic and *n*-heptylic acids, containing five and seven carbons respectively, give rise to glucose, whereas *n*-butyric and *n*-caproic acids, containing four and six carbons respectively, do not give rise to glucose, but to an increase in the acetone bodies. These findings add increased support to Knoop's hypothesis of β -oxidation.

In this communication are recorded the results of experiments

with isobutyric, isovalerianic and isocaproic acids fed to diabetic dogs

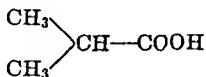
The methods employed were essentially the same as those of the previous experiments, with the exception that the phlorhizin was administered according to the Coolen method. It yields very satisfactory results, and with greater comfort to both operator and animal. The β -hydroxybutyric acid was determined by Shaffer's method.

The iso compounds enumerated above stand in very close relationship to several important amino-acids,



and the results of our experiments seem to throw light on the intermediary metabolism of these amino-acids

The effect of isobutyric acid



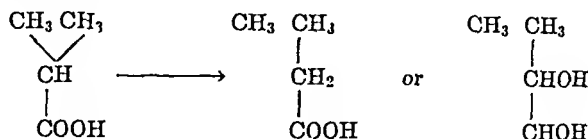
Baer and Blum³ gave 20 grams of isobutyric acid to a diabetic patient with the object of ascertaining whether it would cause increased elimination of the acetone bodies. They did not get any increase in the β -hydroxybutyric acid or acetone. Embden, Salomon and Schmidt⁴ perfused the liver with blood containing isobutyric acid and found no increase in the acetone formation.

In this series of experiments isobutyric acid was fed with a double object in view. First, both Baer and Blum and Embden

³ Baer and Blum *Arch f exp Path u Pharm*, lv, p 89, 1906

⁴ Embden, Salomon and Schmidt *Hofmeister's Beiträge*, viii, p 129

suggested the possibility of demethylation in the catabolism of the isobutyric acid molecule, with the introduction of either an H or an OH in the α -carbon



If this were true, isobutyric acid would give rise to propionic acid or lactic acid, either of which should give rise to extra glucose. Second, it was of great importance to study the possible effects of isobutyric acid on acidosis.

In experiment XI, period VI, the animal was given subcutaneously 10 grams of isobutyric acid as sodium salt. The glucose elimination rose from 11.5 to 14.03 grams. The nitrogen elimination sank from 3.6 in the fore period to 3.29 and 2.55 in periods VI and VII, respectively, to rise again to 3.61 in period VIII. The amount of extra glucose eliminated was 5.52 grams (calculated by the method suggested by Lusk and Ringer).⁵ The most remarkable effect of the isobutyric acid was on the acetone and β -hydroxybutyric acid elimination. In the fore period the acetone elimination was 0.223 gram, it dropped down to 0.059 and rose again to almost the original level in period VII. The β -hydroxybutyric acid, which stood at 1.1 grams for two successive periods, dropped to 0.238 gram and returned to 1.325 grams as the effect of the isobutyric acid wore off. In period XII of the same experiment a similar dose of isobutyric acid was administered to the same dog. The effects were essentially the same, except that the amount of extra glucose was less than in the first case.

In periods IX and XII of experiment XII the animal received 10 grams of isobutyric acid. The yield of extra glucose was 7.6 grams in period IX and 4.3 grams in period XII.

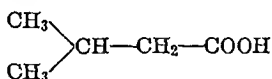
In period VI of experiment XII the animal received 20 grams of isobutyric acid, which resulted in the elimination of 9.15 grams of extra glucose. In period XII of the same experiment the animal was given subcutaneously 10 grams of isobutyl alcohol, which resulted in the elimination of 10.3 grams of extra glucose.

⁵ Ringer and Lusk *Zeitschr f physiol Chem*, lxxvi, p 106, 1910

All these experiments prove beyond question that *isobutyric acid gives rise to considerable quantities of extra glucose*. In most of the cases there was a very marked diminution in the nitrogen output, immediately following the isobutyric acid administration. This phenomenon is very marked in experiments XI, XII and XIV. It is, however, absent in experiment XIII, periods VI and VII.

Another point of great interest is the effect of isobutyric acid on the elimination of acetone bodies. In experiment XI the effect was very marked, while in the others it was hardly noticeable. These individual differences point very strongly to the existence of certain factors in the metabolism of the diabetic organism which determine the action of isobutyric acid and similar substances. Since these experiments were performed, we have administered isobutyric acid to several patients, some with very severe and others with moderately severe acidosis. It was found that in severe cases of diabetes most of the isobutyric acid is converted into glucose, no effect whatsoever being exerted on the acidosis. In the milder cases it is similarly converted into glucose, but very distinct anti-ketogenetic effects are noted. The influence of isobutyric acid on the nitrogen metabolism in animals and on acidosis in human diabetes will form the subject of a separate communication in the near future.

The effect of isovalerianic acid



That isovalerianic acid gives rise to large quantities of β -hydroxybutyric acid, aceto-acetic acid and acetone has been proven beyond question by Baer and Blum⁶ in the diabetic patient and by Embden⁷ who found an increase in the aceto-acetic acid formation on perfusing an excised liver with blood containing 2 grams of isovalerianic acid.

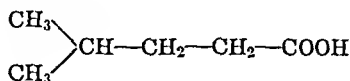
In this research it was our object to see whether isovalerianic acid has any influence on the glucose elimination. In experiment XI period IX, in experiment XII period VI and in experiment XIV

⁶ *Loc cit*

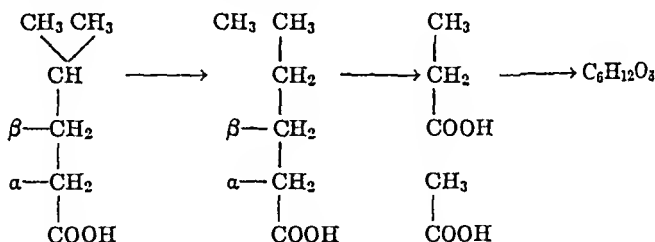
⁷ *Loc cit*

period XI, 10 grams of isovalerianic acid as sodium salt were administered subcutaneously. In none of these experiments was there any increase in the glucose. The elimination of the acetone bodies was increased to a remarkable extent, thus confirming the findings of Baer and Blum and of Embden.

The effect of isocaproic acid (isobutyl acetic acid)



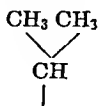
Embden perfused a dog's liver with blood containing isocaproic acid and obtained no increase in the aceto-acetic acid formation. We performed our experiments with this substance to see what influence it has on acidosis and whether it will give rise to extra glucose. Reasoning *a priori*, one would expect this substance to give rise to glucose. The process of demethylation having been established in iso compounds in the case of isobutyric and isovalerianic acids, there is every reason to suppose that such a process takes place in the isocaproic acid molecule. Valerianic acid would be formed first, which after undergoing β -oxidation, would give rise to propionic acid and to glucose.



In period III of experiment XIII, 11.6 grams of isocaproic acid were given subcutaneously as sodium salt. The glucose elimination rose from 24.6 grams in the fore period to 25.19 grams, in spite of the drop in the nitrogen. The D/N ratio, which was 3.38 in the fore period and 3.36 in the after period, rose to 3.65. The extra glucose eliminated was 1.95 grams. In experiments XIV periods XVIII and XIX and in experiment XV period IX, 10 grams of isocaproic acid as sodium salt were given *per os*. The results in both cases corroborate the results in the first experiment in showing

that extra glucose is formed from isocaproic acid. It is true that the amount of extra glucose does not come up to the theoretical value, but, as will be seen from other experiments, theoretical yields are not always obtained.

Intermediary metabolism of fatty acids containing an isopropyl radical



From the work of Baer and Blum, Embden and our own, it is apparent that all compounds containing an isopropyl radical undergo demethylation. The question of interest in this connection is, in what way does the methyl radical dissociate from the rest of the molecule? Baer and Blum have advanced the suggestion that the CH_3 may leave the molecule and an OH radical take its place. They based this suggestion upon the fact that after feeding 20 grams of isobutyric acid they obtained 0.5 gram of zinc lactate from one-half of the twenty-four hours' urine, and also upon the fact that isovalerianic acid gave rise to β -hydroxybutyric acid. We are inclined to believe that the methyl radical is removed by a process of hydrolysis, the OH going to the methyl radical, forming CH_3OH , which is oxidized in the body, while the H radical goes to replace the CH_3 , forming an acid of the normal series. Thus isobutyric acid gives rise to propionic acid which in turn may give rise to lactic acid. Isovalerianic acid gives rise to butyric acid which in turn produces β -hydroxybutyric acid. This also explains why Baer and Blum obtained β -hydroxybutyric acid from α -methylbutyric acid and failed to get it from α -hydroxybutyric acid.

Intermediary metabolism of amino-acids containing an isopropyl radical

Embden and Marx⁸ in their perfusion experiments found that α -amino-valerianic acid gave rise to aceto-acetic acid, while neither α -amino-butyric nor α -amino-caproic acids showed any evidence of

⁸ Embden and Marx *Hofmeister's Beiträge*, vi, p. 318, 1908

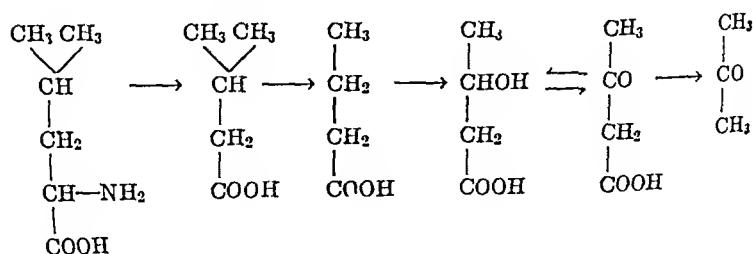
yielding aceto-acetic acid. They drew the conclusion that α -amino-acids suffer oxidation in the α -position giving rise to acids with one carbon atom less, while the carboxyl radical is split off, in all probability in the form of CO_2 .

In the protein molecule we find two amino-acids which contain the isopropyl radical—leucine and valine. Halsey⁹ fed leucine to phlorhizinated dogs, and, of six feedings, two showed considerable amounts of extra glucose, and four gave entirely negative results. Recently Dakin published one experiment in which he fed 15 grams of α -leucine and obtained 4 grams of extra glucose. Neither Halsey nor Dakin are inclined to attribute glucogenetic properties to leucine.

When we consider leucine in the light of our experiments with unsubstituted fatty acids with branched chains, and having in mind Embden's results, in which he showed that leucine is a strong aceto-acetic acid builder, there remains only one possible conclusion with regard to the catabolism of leucine. As far as our knowledge goes, and as will be shown in a following communication, we are fully justified in assuming that all carbons in the α -position having an amino radical lose the amino radical and become oxidized possibly to the carboxyl state, giving rise to compounds (acids) with one carbon less.

For the present we do not include glycocoll or alanine in this category. Experiments are in progress which will throw light on the rôle of the carboxyl in the formation of glucose from these substances.

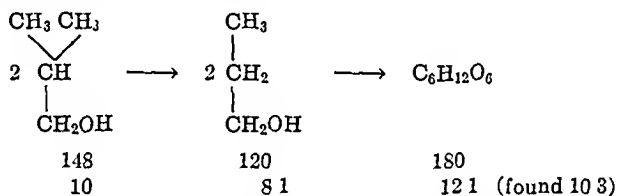
Leucine in its catabolism can be assumed to pass through the following intermediary stages



⁹ Halsey *Amer Journ of Physiol*, x, p 229, 1903

Ordinarily leucine cannot be classed as a glucose-yielding substance, but under certain circumstances it may give rise to glucose. There are certain flora of intestinal bacteria that seem to possess the power of effecting α -oxidation. In one of our experiments butyric acid, which, when given subcutaneously never yields even a trace of glucose, when fed *per os* gave rise to as much as 3 grams of glucose. This reaction cannot be explained in any other way, and it seems very possible that the same factors obscured Halsey's two experiments.

When we found that isobutyric acid gave rise to glucose and that isobutyl alcohol could yield an amount of glucose that corresponds to the conversion of three of its carbons into glucose, we were led to the belief that valine in its catabolism was broken down to isobutyric acid, and that it would be found to be one of the amino-acids that give rise to glucose.



In his paper on the "Intermediary Metabolism of Amino-acids," Dakin¹⁰ showed that valine yields practically no glucose. This of course makes our theory more difficult of interpretation, but we do not believe it robs the theory of its strength, for the antiketogenic properties of valine are very similar to those of isobutyric acid, and as will be shown in the future, this property is possessed only by compounds capable of forming glucose. (The reverse, however, is not true, *i.e.*, not all compounds that are capable of forming glucose possess antiketogenic properties.)

SUMMARY

Experiments were performed on phlorhizinized dogs.

I. It was found that isobutyric acid and isobutyl alcohol give rise to glucose, probably by undergoing demethylation and by giving rise to normal fatty acids (propionic acid).

¹⁰ Dakin. *This Journal*, *xiv*, p. 321, 1913.

II Isovalerianic acid does not give rise to glucose, but to large quantities of aceto-acetic acid, acetone and β -hydroxybutyric acid

III Isocaproic acid was found to give rise to glucose, probably having, by a process of demethylation, formed normal valerianic acid, which became oxidized to propionic acid

IV Isobutyric acid, in certain cases, possesses very marked anti-ketogenetic properties

V It is suggested that isovalerianic acid is one of the intermediary stages in the catabolism of leucine

VI It is also suggested that isobutyric acid may be an intermediary body in the catabolism of valine

EXPERIMENT XI Twelve-hour periods

DATE 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL DEXTROSE	POLARIZATION	D N	EXTRA DEXTROSE	NH ₃ N	ACETONE AND ACETIC ACID	B-HYDROXY-BUTYRIC ACID	REMARKS
Feb 27	III		3 96	13 76	+0 63°	3 47		0 153	0 128	0 509	1 gm phlorhizin
27	IV	8 66	3 45	11 21	+0 50°	3 26		0 226	0 197	1 12	
28	V		3 60	11 50	+0 52°	3 20		0 274	0 223	1 165	1 gm phlorhizin
28	VI	8 32	3 29	14 03	+0 66°	4 26		0 187	0 059	0 238	10 gms isobutyric acid given subcutaneously as Na salt in 2 doses
March 1	VII		2 55	10 44	+0 45°	4 11	5 52	0 134	0 210	1 325	1 gm phlorhizin
1	VIII	8 26	3 36	11 05	+0 46°	3 29		0 266			
2	IX		3 61	11 47	+0 44°	3 18		0 334	0 549	2 75	1 gm phlorhizin 10 gms isovalerianic acid
2	X	8 08	3 24	10 75	+0 41°	3 32		0 218	0 37	2 50	
3	XI		3 31	10 52		3 18		0 266	0 183	1 14	1 gm phlorhizin
3	XII		3 18	12 42	+0 54°	3 91	2 55	0 100	0 059	0 237	10 gms isobutyric acid dissolved in 30 cc of water, given subcutaneously as Na salt in 2 doses
4	XIII		2 60	7 88		3 03		0 157	0 076	0 671	1 gm phlorhizin
4	XIV	7 82	2 30	7 62		3 31		0 142	0 219	1 29	
5	XV		2 68	8 35		3 11		0 204	0 262	1 57	1 gm phlorhizin

EXPERIMENT XII Twelve-hour periods

DATE 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZA- TION	D N	EXTRA GLUCOSE	NH ₃ N	ACETONE AND ACETO- ACID	β HYDROXY- BUTYRIC ACID	REMARKS
Feb 27	III		5 14	18 93	+0 85°	3 69		0 555	0 211	0 830	1 gm phlorhizin
27	IV	12 70	1 60	16 90	+0 76°	3 08		0 434	0 287	1 598	1 gm phlorhizin
28	V		5 02	17 51	+0 765°	3 49		0 198	0 328	2 44	10 gms isovalerianic acid
28	VI	12 30	3 98	15 55	+0 53°	3 01		0 511	0 937	5 81	1 gm phlorhizin
March 1	VII		4 83	17 86	+0 75°	3 70		0 714	0 574	3 58	1 gm phlorhizin
1	VIII	12 30	4 30	16 37	+0 72°	3 81		0 565	0 410	1 95	1 gm phlorhizin 10 gms isobutyric acid, subcutaneously
2	IX		3 28	17 50	+0 73°	5 34	7 60	0 311	0 281	1 89	1 gm phlorhizin
2	X	12 38	3 09	14 85	+0 68°	4 82		0 230	0 395	1 29	1 gm phlorhizin
3	XI		3 74	14 84		3 96		0 470	0 606	2 16	10 gms isobutyric acid given subcutaneously as Na salt in 2 doses
3	XII	12 08	2 93	13 86	+0 625°	4 76	4 31	0 180	0 642	1 95	1 gm phlorhizin
4	XIII		2 96	12 20		4 13		0 174	0 464	1 50	1 gm phlorhizin
4	XIV	11 06	3 44	11 70		3 49		0 214	0 370	1 145	1 gm phlorhizin
5	XV		3 34	10 49		3 14		0 332	0 454	1 74	1 gm phlorhizin

EXPERIMENT XIII Twelve-hour periods

DATE 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZATION	D N	ETHYL GLUCOSE	NH ₃ N	ACETONE AND ACETIC ACID	β HYDROXY-BUTYRIC ACID	REMARKS
March											
12	II		7 28	24 60	+1 158°	3 38		0 587	0 311	2 182	1 gm phlorhizin
13	III	15 30	6 90	25 19	+1 20°	3 65	1 95	0 308	0 259	1 087	11 6 gms isocaproic acid given subcutaneously as Na salt
13	IV		7 14	23 95	+1 15°	3 36	9 15	0 383	0 233	0 922	1 gm phlorhizin
14	V	15 00	6 98	22 26	+0 96°	3 19		0 505	0 297	1 254	
14	VI		6 87	27 90	+1 30°	4 06		0 278	0 299	1 43	1 gm phlorhizin 20 gms isobutyric acid given subcutaneously as Na salt in 2 doses
15	VII		7 27	25 65	+1 21°	3 53	10 28	0 210	0 320	0 821	
15	VIII	14 52	7 61	23 48	+1 02°	3 09		0 415	0 350	0 924	1 gm phlorhizin
16	IX		6 87	21 18	+1 07°	3 07		0 325	0 347	0 992	
16	X		5 98	23 09	+0 90°	3 85	10 28	0 292	0 280	0 694	1 gm phlorhizin
17	XI	13 72	5 25	20 14	+0 86°	3 84		0 265	0 149	0 383	
17	XII		4 50	21 38	+0 86°	4 75		0 293	0 135	0 477	1 gm phlorhizin 10 gms isobutyl alcohol given subcutaneously
18	XIII	13 12	4 09	20 40	+0 905°	5 00	10 28	0 366	0 185	0 542	
18	XIV		5 74	20 09	+0 86°	3 50		0 364	0 178	0 520	1 gm phlorhizin

EXPERIMENT XIV Twelve-hour periods

DATE 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZATION	D N	EXTRA GLUCOSE	NH ₃ N	ACETONE AND ACETO-ACETIC ACID	β HYDROXY BUTYRIC ACID	REMARKS
March 16	X		4.93	19.45	+0.83°	3.95		0.253	0.219	0.55	1 gm phlorhizin
17	XI	11.20	5.42	18.55	+0.77°	3.42		0.359	0.55	2.39	10 gms isovaleric acid given subcutaneously as Na salt in 1 dose
17	XII		5.61	18.59	+0.78°	3.31		0.346	0.455	2.58	1 gm phlorhizin
18	XIII	10.68	6.46	21.46	+0.895°	3.35		3.342	0.314	1.66	
18	XIV		5.80	19.70	+0.805°	3.40		0.439	0.330	2.24	1 gm phlorhizin
19	XV		5.17	20.67	+0.875°	4.00	3.21	0.252	0.273	1.79	10 gms isobutyric acid given per os as Na salt
19	XVI*	{	4.78	16.05	+0.613°	3.36		0.515	0.435	2.87	1 gm phlorhizin
20	XVII		4.78	16.05	+0.613°	3.36		0.515	0.435	2.87	
20	XVIII		10.22	14.94	+0.615°	3.16		0.695	0.331	1.93	1 gm phlorhizin
21	XIX	9.69	3.45	13.29	+0.565°	3.27	2.78	0.270	0.303	1.29	10 gms isocaproic acid given per os as Na salt
21					+0.512°	3.85			0.333		1 gm phlorhizin

Analysed together

EXPERIMENT XV Twelve-hour periods

DATE 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZATION	D N	EXTRA GLUCOSE	NH ₃ N	ACETONE AND ACETO-ACETIC ACID	β HYDROXY BUTYRIC ACID	REMARKS
March 30	VIII	7.90	2.80	9.14	+0.378°	3.37			0.549	1.935	1 gm phlorhizin given subcutaneously
30	IX		2.83	10.51	+0.380	3.71			0.700	2.030	10 gms isocaproic acid given per os as Na salt
31	X					3.84	Some urine lost				

THE CHEMISTRY OF GLUCONEOGENESIS ¹

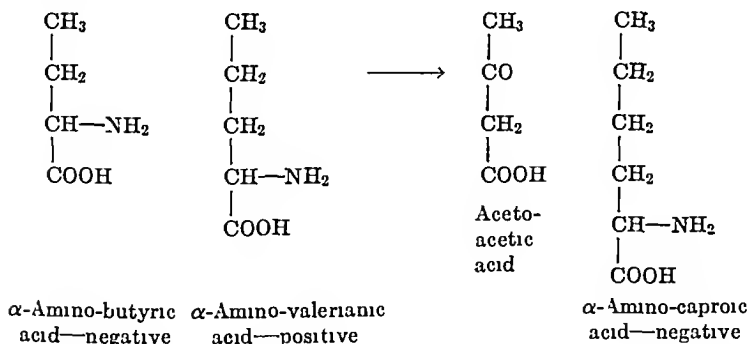
IV THE FATE OF SUCCINIC, MALIC AND MALONIC ACIDS IN THE DIABETIC ORGANISM, WITH CONSIDERATION OF THE INTERMEDIARY METABOLISM OF ASPARTIC AND GLUTAMIC ACIDS, PROLINE, LYSINE, ARGININE AND ORNITHINE

BY A I RINGER, E M FRANKEL AND L JONAS

(From the Department of Physiological Chemistry of the University of Pennsylvania, Philadelphia, Pa.)

(Received for publication, April 30, 1913)

Embden and Marx² found that on perfusing an extirpated liver with blood to which α -amino-valerianic acid had been added, there was an increase in the aceto-acetic acid formation, while α -amino-butyric acid and α -amino-caproic acid gave negative results



It is evident that α -amino-valerianic acid must have been changed to a four-carbon compound before it could possibly give rise to aceto-acetic acid. The authors therefore concluded that in α -amino-acids the α -carbon, containing the amino radical, becomes

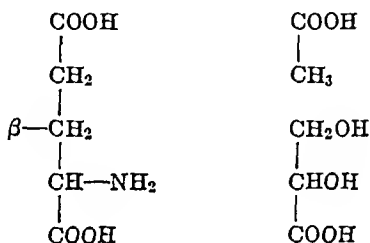
¹ Aided by a grant from the Rockefeller Institute for Medical Research

² Embden and Marx *Hofmeister's Beiträge*, vi, p 318, 1908

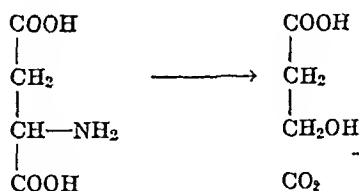
oxidized to a carboxyl state, giving rise to a fatty acid with one carbon less

Lusk³ found that the feeding of 20 grams of glutamic acid was followed by an elimination of 13.5 grams of extra glucose

Ringer and Lusk⁴ extended these investigations and found that 20 grams of aspartic acid yielded as much as 14.9 grams of extra glucose. These results corresponded to the conversion into glucose of three of the carbons of either the aspartic or glutamic acid molecule. It was then suggested that glutamic acid undergoes the following changes in the diabetic organism by giving rise to glyceric acid, which was found capable of forming extra glucose



For aspartic acid it was suggested that the molecule may suffer oxidation in the α -position, i.e., in the carbon containing the amino radical, giving rise to hydracrylic acid

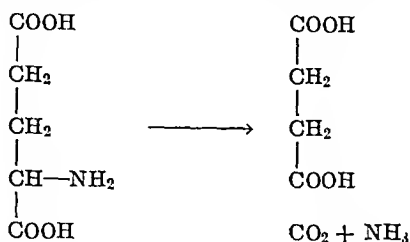


In this series of researches it was our object to test experimentally the paths that these amino-acids may take in their catabolism. In view of the outcome of Embden and Marv' experiments, it suggested itself that glutamic acid may undergo deamination with oxidation in the α -carbon giving rise to a

³ Lusk, *Amer Journ of Physiol*, xxii, p 174, 1908

⁴ Ringer and Lusk, *Zeitschr f physiol Chem*, lxi, p 106, 1910

dibasic acid with one less carbon, *i e*, with four carbons, namely, succinic acid. If this were true, succinic acid would yield glucose in the diabetic animal as readily as does glutamic acid



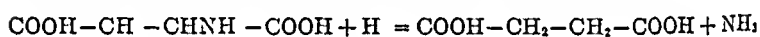
In experiment XVI, period IV, 11.8 grams ($\frac{N}{10}$) of succinic acid as sodium salt were given *per os*. The glucose elimination, which was 27.26 in the fore period and 25.02 in the after period, rose to 32.74 grams. The D/N ratio rose from 3.41 to 3.99. The amount of extra glucose eliminated was 5.15 grams. In experiment XVII, period XIII, 11.8 grams of succinic acid as sodium salt were given subcutaneously. The yield of extra glucose was much larger, amounting to 9.45 grams.

These two experiments prove very conclusively that succinic acid can yield large quantities of extra glucose. The question now arises—is it merely an incident that these two substances give rise to glucose or does it actually *prove* that glutamic acid passes through succinic acid in its intermediary stages of metabolism? We believe that the latter is the case, and that glutamic acid *does* give rise to succinic acid for the following additional reason. Succinic acid is found as one of the by-products of alcoholic fermentation. Pasteur⁵ proved its presence conclusively and found that the quantity of succinic acid bore a relationship of 0.4 to 0.7 per cent to the fermented glucose. He believed that succinic acid was a product of the fermentation of the glucose molecule. This theory of Pasteur greatly disturbed the then current conception of Gay-Lussac that a molecule of glucose, in the process of fermentation, breaks down to two molecules of alcohol and two molecules of carbon dioxide.



⁵ Pasteur *Compt rend Acad Sci*, 1858 to 1859, *Ann de chim et de phys* (3) lvi, p 323, 1860

'During the next forty years this was the subject of a great many investigations and slowly evidence accumulated which tended to disprove Pasteur's theory of succinic acid arising from the glucose molecule. It was shown that the quantity of succinic acid was not proportional to the amount of glucose fermented, but to the length of time that fermentation was permitted to go on. Buchner and Meisenheimer⁶ finally succeeded in showing that succinic acid was not a product of glucose fermentation, but a product of the metabolism of the yeast cell. Working with expressed cell-free juice of yeast, they could find no succinic acid. The problem of the origin of succinic acid in the by-products of alcoholic fermentation was solved definitely by Felix Ehrlich.⁷ He found that the higher alcohols of fusel oil were the products of the protein metabolism of the yeast cell. He then devoted his researches to finding the mother substances of succinic acid. It was already known at that time that bacteria were capable of effecting deamination of amino-acids by splitting off the NH_2 radical and converting it into NH_3 , substituting a hydrogen for the removed NH_2 .



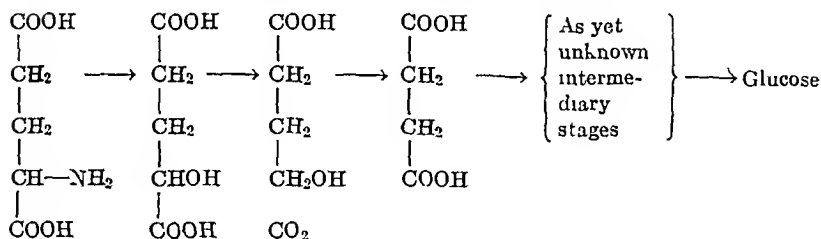
It was also known that in addition to succinic acid small quantities of aspartic acid were found in the fermentation mixture. It therefore suggested itself to Ehrlich that aspartic acid might be the mother substance of succinic acid and that glutamic acid might be the mother substance of glutaric acid, which would mean, if proven, that yeast cells, like bacteria, are capable of bringing about deamination in a very simple way—by splitting off NH_2 and substituting a hydrogen for it. When he came to subject these ideas to the test of experimentation, he found that the addition of aspartic acid to fermenting yeast and sugar was followed by no increase in the succinic acid, but when glutamic acid was added, he was able "*stets sehr beträchtliche, den normalen Gehalt weit übersteigende Mengen von Bernsteinsäure, zu isolieren*"

Here we see the direct transformation of glutamic acid into succinic acid by a living cell, and when we compare these results

⁶ Buchner and Meisenheimer *Ber d deutsch chem Gesellsch*, xxix, p 3201, 1906, xxiv, p 1529, 1901

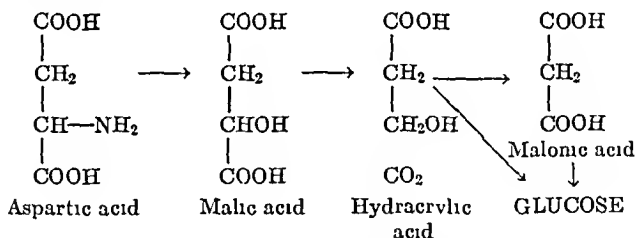
Ehrlich *Biochem Zeitschr*, xviii, p 391, 1909

with our own in the diabetic organism, we feel fully justified in concluding that *the path of glutamic acid in metabolism is through succinic acid* undoubtedly passing through α -hydroxy-glutaric acid and γ -hydroxy-butyric acid as intermediary stages



The fate of aspartic acid in the diabetic organism

In the foregoing it was shown that α -amino-glutaric acid (glutamic acid) becomes catabolized to succinic acid, which in turn gives rise to extra glucose. A similar path of catabolism suggests itself for aspartic acid, which is chemically very closely related to glutamic acid.



Aspartic acid, as was shown by Ringer and Lusk,⁸ and asparagine, as was shown by Knopf,⁹ can give rise to large quantities of glucose. In tracing the possible intermediary compounds, one should find all of them capable of yielding glucose to the same extent as does aspartic acid. The first intermediary product of aspartic acid after deamination appears to be malic acid. In experiment XVI, period II, 13.4 grams ($\frac{M}{176}$) of malic acid as sodium salt were administered subcutaneously. The amount of glucose

⁸ Ringer and Lusk *loc cit*

⁹ Knopf *Arch f exp Path u Pharm*, *vol*, p 123, 1903

elimination rose considerably, but not all of it can be attributed to the malic acid, because there was a considerable rise in the protein metabolism of the same period. The D N ratio rose from 3.4 in the fore period to 3.97 in the experimental period. The amount of extra glucose eliminated was 5.94 grams. Very convincing results were obtained in experiment XX, period VII. 13.4 grams of malic acid, neutralized with calculated amounts of sodium and potassium hydroxide, were given *per os*. The glucose elimination rose from 14 grams in the fore period to 21.14 grams, in spite of the drop of the nitrogen metabolism. The D N ratio rose from 3.12 to 5.27 and returned in the after periods to 3.27 and 3.22. The calculated extra glucose amounted to 8.32 grams.

These two experiments prove very conclusively that malic acid gives rise to glucose to approximately the same extent as does aspartic acid, and that it may be considered a product of aspartic acid metabolism.

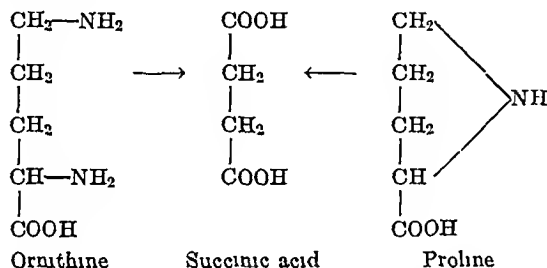
The next question to decide was whether hydracrylic acid and malonic acid gave rise to glucose. Unfortunately we had none of the former in our possession and were forced to postpone the experiment to a future date. Malonic acid was administered seven times to five different dogs. It was found that because of the large quantities of alkali necessary to neutralize the acid, it was absorbed with great difficulty, and we were never sure that all of the administered material was absorbed. In some cases it was administered *per os*, in others subcutaneously. The oral administrations were always followed by diarrhoea. The amounts of extra glucose produced by malonic acid are here tabulated.

Experiment	Period	II	Extra Glucose	grams
XVII	"	XVI	"	1.00
XVIII	"	IV	"	2.64
XIX	"	VII	"	0.65
XX	"	X	"	2.84
XXI	"	III	"	3.06
XXII	"	III	"	1.00

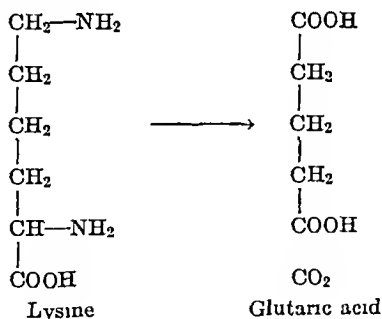
The results are very low indeed, but it is very likely that in the transformation of aspartic acid to a compound with three carbons, the reaction ordinarily does not proceed to a point of complete oxidation of the terminal carbon. It is very likely that hydracrylic acid goes over into glucose before the alcohol radical becomes oxidized to a carboxyl.

Intermediary metabolism of lysine, arginine, ornithine and proline

In a recent series of papers, Dakin¹⁰ showed that arginine, ornithine and proline give rise to glucose, when fed to diabetic dogs, while lysine does not give rise to glucose. Dakin rightly suggests that of the arginine only the ornithine moiety goes over into glucose. It seems to us that ornithine and proline give rise to glucose because of their ability to form succinic acid after undergoing deamination.



Lysine, on the other hand, after undergoing deamination, becomes converted into glutaric acid and this was shown not to be convertible into glucose.



SUMMARY

Experiments were performed on phlorhizimized animals

I It was found that succinic, malic and perhaps also malonic acids give rise to extra glucose

¹⁰ Dakin this *Journal*, *xiii*, p 513, 1913, *xiv*, p 321, 1913

II Evidence was presented to the effect that succinic acid is an intermediary body in the metabolism of glutamic acid, ornithine and proline, which accounts for the conversion of these substances into glucose

III It is suggested that malonic acid may arise in part from the catabolism of aspartic acid

IV It was also suggested that lysine in its catabolism passes through a glutaric acid stage, which accounts for its non-conversion into glucose

EXPERIMENT XVI Twelve-hour periods

DATE	WEIGHT	PERIOD	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZATION	D N	EXTRA GLUCOSE	NH ₃ N	TOTAL ACETOYE	REMARKS
Oct 30		I	8 35	28 37	1 096°	3 40		0 56	0 28	
30	14 49	II	10 29	40 92	1 636°	3 97	5 94	0 29	0 33	13 4 gms malic acid as sodium salt dissolved in water given subcutaneously
31		III	7 99	27 26	1 150°	3 41		0 33	0 25	
31		IV	8 19	32 74	1 402°	3 99	5 15	0 20		11 8 gms succinic acid as sodium salt given <i>per os</i>
Nov 1		V	7 49	25 02		3 34		0 37	0 367	

EXPERIMENT XVII Twelve-hour periods

DATE	PERIOD	TOTAL NITROGEN	TOTAL GLUCOSE							REMARKS
Nov 12										
14	I	3 72	14 38		3 86			0 19	0 56	
14	II	4 20	14 84		3 53		0 0	0 24	0 95	10 4 gms malonic acid as sodium salt dissolved in water given subcutaneously
15	III	3 90	14 79		3 79			0 22	0 35	

EXPERIMENT XVIII Twelve-hour periods

DATE	WEIGHT	PERIOD	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZA- TION	D	EXTRA GLUCOSE	NH ₃ N	ACETO- LYACETIC ACID	β HYDROXY- BUTYRIC ACID	REMARKS
April 1913											
7		I	6 14	17 85	0 80°	2 92		0 53	0 47	1 16	
7	12 53	II	5 53	16 84	0 85°	3 05		0 46	0 41	1 14	
8		III	5 35	24 30	1 13°	4 55	9 45	0 25			11 8 gms succinic acid as sodium salt dis- solved in water, given subcutaneously
8	12 46	IV	4 47	16 87	0 80°	3 78		0 28	0 37	1 46	
9		V	4 53	15 43	0 72°	3 41		0 33	0 48	1 73	
9	12 71	VI	4 20	15 95	0 78°	3 80	1 00	0 30	0 45	1 85	10 4 gms malonic acid as sodium salt given per os Diarrhoea
10		VII	3 05	11 21	0 62°	3 68		0 22	0 41	1 56	
10	12 32	VIII	3 11	11 76	0 50°	3 78			0 56	2 34	

EXPERIMENT XIX Twelve-hour periods

DATE	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	POLARIZA- TION	D N	EXTRA GLUCOSE	NH ₃ N	ACETONE AND ACETO- ACETIC ACID	β HYDROXY- BUTYRIC ACID	REMARKS
April 1913											
6	I		4.34	14.22	0.625°	3.28		0.27	0.15	0.39	
7	II		5.10	16.55	0.88°	3.24		0.30	0.22	0.54	
7	III		4.99	16.05	0.75°	3.24		0.28	0.25	0.79	
8	IV	6.80	5.21	19.82	0.87°	3.81	2.84	0.23	0.41	1.56	10.4 gms malonic acid as sodium salt given per os in one dose One watery movement of bowels
8	V		5.08	17.36	0.80°	3.42		0.29	0.52	1.72	
9	VI	6.45	4.99	16.86	0.73°	3.38		0.33	0.48	1.87	
9	VII		4.93	17.45	0.78°	3.54	0.65	0.30	0.45	0.98	10.4 gms malonic acid as sodium salt dissolved in water given subcutaneously
10	VIII	6.43	4.67	16.05	0.73°	3.44		0.27	0.17	0.51	
10	IX		5.28	16.95	0.75°	3.21		0.29	0.17	0.52	
11	X	6.25	2.88	11.83	0.55°	4.11	2.84	0.13	0.12	0.30	10.4 gms malonic acid as sodium salt given per os Diarrhoea
11	XI		3.79	11.46	0.52°	3.03		0.17	0.21	0.63	
12	XII		3.39	9.60		2.83		0.32	0.10	0.26	

EXPERIMENT XX Twelve-hour periods

DATE	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	PLASMA TIDN	D N	EXTRA GLUCOSE	NH ₃ N	TOTAL ACETONE	PERFORMED ACETONE	ACETO-ACETIC ACID	β HYDROXY ACID	REMARKS
April 1913													
14	I	13 40	7 99	29 35	1 26°	3 67		0 46	0 310	0 061	0 249	1 01	10 4 gms malonic acid as sodium and potassium salt given subcutaneously in two doses
15	II		8 13	30 41	1 39°	3 74		0 52	0 365	0 059	0 306	1 28	
15	III	13 09	8 20	33 86	1 50°	4 13	3 06	0 53	0 466	0 061	0 406	1 72	
16	IV		9 08	34 39	1 60°	3 79		0 58	0 457	0 083	0 374	1 74	
16	V	12 01	9 42	36 38	1 62°	3 86		0 64	0 554	0 028	0 526	2 39	
17	VI		7 49	28 25	1 32°	3 78		0 47					

EXPERIMENT XXI Twelve-hour periods

DATE	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	PLASMA TIDN	D N	EXTRA GLUCOSE	NH ₃ N	TOTAL ACETONE	PERFORMED ACETONE	ACETO-ACETIC ACID	β HYDROXY ACID	REMARKS
April 15	II		4 43	12 50	0 60°	2 82		0 25	0 206	0 129	0 077		10 4 gms malonic acid as sodium and potassium salt dissolved in water given subcutaneously in two doses
15	III	10 39	4 93	15 64	0 72°	3 17	1 00	0 32	0 216	0 076	0 137	0 792	
16	IV		4 81	14 90	0 61°	3 10		0 32	0 283	0 087	0 196	1 32	
16	V	10 21	4 32	14 32	0 63°	3 32		0 36	0 271	0 081	0 190	1 86	
17	VI		4 50	14 00	0 60°	3 12		0 45	0 295	0 066	0 239	1 96	13 4 gms malic acid as sodium and potassium salt given per os
17	VII	9 94	4 02	21 14	1 04°	5 27	8 32	0 08	0 313	0 126	0 188	1 23	
18	VIII		3 96	12 92	0 49°	3 27		0 32	0 434	0 123	0 371	2 66	
18	IX	9 77	4 20	13 51	0 60°	3 22		0 31	0 500	0 202	0 201	2 28	

ON THE ACTION OF LEUCOCYTES ON HEXOSES

FOURTH COMMUNICATION

ON THE MECHANISM OF LACTIC ACID FORMATION

BY P A LEVENE AND G M MEYER

(*From the Rockefeller Institute for Medical Research, New York*)

(Received for publication, April 30, 1913)

It was reported in a previous communication that by the action of leucocytes on *d*-glucose, *d*-mannose or *d*-fructose, *d*-lactic acid was obtained. The mechanism of this transformation may be interpreted by a process consisting of at least three phases. First, transformation of the six carbon chain molecule into two three carbon chains such as glyceric aldehyde, second, into the formation of a substance which would permit a change in the configuration of the groups attached to the α -carbon atom (to the carbonyl group), and third, the transformation of the latter substance into lactic acid. It is evident that the formation of methyl glyoxal in the second phase of the reaction could lead the way to lactic acid formation by enzymes analogous to those which Knoop and Dakin found capable of transforming keto-acids into hydroxy-acids. At the moment when the previous communication was prepared for publication, evidence of the existence of such enzymes was lacking, hence the question was referred to as one requiring further experimental investigation. In fact, experiments in that direction were initiated immediately after the observations reported in the previous communication had been completed.

Simultaneously with our observation on the transformation of the three hexoses into *d*-lactic acid, Dakin and Dudley¹ made the very important discovery of the presence in animal tissues of an enzyme capable of transforming phenyl glyoxal into the natural mandelic acid and later they supplemented it by an observation on the transformation of methyl glyoxal into lactic acid.

¹ Dakin and Dudley this *Journal*, **xiv**, p 155, 1913

The observations of Dakin and Dudley were soon corroborated by Neuberg,² who observed the transformation of pyruvic aldehyde into lactic acid. It seemed at first sight that these observations entirely established the correctness of the view of the mechanism of the transformation of hexoses into lactic acid expressed in our previous communication. However, the conclusion was not absolutely compelling for the following reasons.

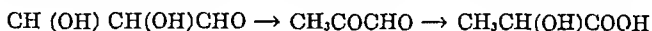
First, under the conditions of the experiments of Dakin and Dudley and of Neuberg, hexoses are not converted into lactic acid, but remain intact, and it does not naturally follow that an enzyme present in autolyzing tissues also is present in the uninjured cells. Second, neither Dakin and Dudley nor Neuberg make positive statements regarding the absolute absence of bacterial growth in their solutions. The presence or absence of bacterial contamination is an all important factor for the consideration of the extent to which the observations of Dakin and Dudley and of Neuberg can be applied to the explanation of the mechanism of conversion of the *d*-hexoses into *d*-lactic acid. The enzymes active in the conversion of hexoses into lactic acid bring about the formation of only the dextro-rotatory substance. In the experiments of Neuberg a mixture of *dl* and of the *d* forms was obtained. It is not excluded, under such conditions, that by the action of the enzyme only the racemic acid was formed and that the dextro-rotatory isomer owed its origin to bacterial action. At the time of Dakin and Dudley's first communication the authors brought no evidence of the formation of the optically active acid. Third, in the work of Neuberg there is a lack of evidence that the *d*-lactic acid was not derived from the liver tissue employed in his experiment.

By all this we do not for a moment intend to detract from the importance of the discovery of Dakin and Dudley and of the observations of Neuberg. However, we felt that in order to establish our first view on the mechanism of lactic acid formation from hexoses beyond dispute, it was imperative to demonstrate the conversion of methyl glyoxal into *d*-lactic acid under the same conditions which enabled us to bring about the conversion of *d*-hexoses into *d*-lactic acid. For these reasons, and with the consent of Dr Dakin,

² Neuberg, *Biochem Zeitschr*, **xlii**, p. 502

we continued our experiments which were in progress at the time of his first publication

Our experiments were performed under exactly the same conditions as those for the conversion of hexoses into lactic acid. There was an absolute absence of bacterial growth, both aerobic and anaerobic. Leucocytes and kidney tissue were employed in the experiments. The conversion of methyl glyoxal into lactic acid took place with either of the tissues. The lactic acid was a mixture of *dl* and of *d* forms. This furnishes convincing evidence to the view that the formation of *d*-lactic acid from the various *d*-hexoses is conditioned by the intermediate formation of methyl glyoxal



Controls with leucocytes alone and with kidney tissue alone (in quantities equal to those employed in the principal experiments) showed the absence of lactic acid

EXPERIMENTAL

Leucocytes These were obtained from dogs by injecting turpentine into the pleural cavity, according to the method previously described

Tissues Rabbits were killed by exsanguination and the kidneys removed aseptically, and minced as fine as possible under sterile conditions

Methyl glyoxal This was prepared from methyl glyoxal-diacetol according to Meisenheimer³. The latter compound was obtained by the method of A. Wohl and M. Lange⁴

Solutions The methyl glyoxal (about 1 gram) was dissolved in 10 cc. of distilled water and sterilized by passing it through a Berkefeld filter. The washed leucocytes were suspended in a 1 per cent Henderson phosphate mixture to which had been added calcium phosphate, 3 grams to each 100 cc. of solution. The sterile methyl glyoxal solution was added last. The same procedure was adopted in the experiment with the kidneys

Lactic acid The mixture of methyl glyoxal with leucocytes

³ Meisenheimer *Ber d deutsch chem Gesellsch*, 4v, p 2635, 1912

⁴ A. Wohl and M. Lange *Ber d deutsch chem Gesellsch*, 41, p 3612, 1908

and kidneys was set aside at 37° for eighteen hours, and then coagulated by heating and with the addition of sufficient phosphoric acid to dissolve the calcium phosphate. The filtered solution was extracted with ether in a von der Heide extracting apparatus after the addition of sodium sulphate and 5 cc of phosphoric acid. The zinc salt was prepared according to the method previously described.

Bacteriological examination Both aerobic and anaerobic cultures were made of the solutions prior to their coagulation by Dr J Bronfenbrenner, and we desire to express our appreciation for his assistance. No bacterial growth was obtained in either instance after forty-eight hours.

EXPERIMENT 1 *Methyl glyoxal and leucocytes* About 25 cc of washed leucocytes were suspended in 100 cc Henderson phosphate solution with 3 grams of calcium phosphate and about 1 gram methyl glyoxal, and allowed to stand for eighteen hours at 37° . There was obtained 0.283 gram zinc lactate.

0.0811 gram of the recrystallized anhydrous salt after ignition gave 0.0275 gram ZnO = 33.80 per cent ZnO. Calculated = 33.40 per cent.

0.2540 gram zinc lactate in 5 cc water, total weight 5.3622 grams, gave a rotation in a 2 dm tube of $\alpha = -0.14^{\circ}$ (pure D-light).

EXPERIMENT 2 *Methyl glyoxal and kidneys* Both kidneys of a rabbit were minced and added to 100 cc Henderson phosphate solution containing 3 grams of calcium phosphate, and about 1 gram of methyl glyoxal in 10 cc water added. There was obtained 0.2440 gram zinc lactate.

0.0598 gram of the anhydrous salt after ignition gave 0.0198 gram ZnO = 33.28 per cent ZnO. Calculated = 33.40 per cent.

0.2440 gram zinc lactate dissolved in 5 cc water, total weight 5.8350 grams, gave a rotation in a 2 dm tube of $\alpha = 0.10^{\circ}$ (pure D-light).

A CONTRIBUTION TO A THEORY CONCERNING THE INTERMEDIARY METABOLISM OF CARBO- HYDRATES AND PROTEINS

THE MUTUAL INTERCONVERSION OF α -AMINO-ACIDS, α -HYDROXY-ACIDS AND α -KETONIC ALDEHYDES

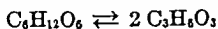
BY H D DAKIN AND H W DUDLEY

(*From the Herter Laboratory, New York*)

(Received for publication, May 2, 1913)

The object of the following communication is to give a preliminary account of certain new observations that appear to be of significance in connection with the intermediary metabolism of carbohydrates and of proteins¹

It has long been known that lactic acid occupies an exceptional position in relation to the metabolism of glucose and of amino-acids. Its formation from glucose and glycogen has been accepted for many years and the work of Embden and his collaborators² demonstrating its production during the perfusion of glycogen-rich livers afforded strong support for this view. But the first really convincing evidence of the decomposition of glucose with a formation of lactic acid commensurate with the disappearance of glucose was furnished by the admirable experiments of Levene and Meyer³ on the action of leucocytes upon sugars. The converse change, namely, the conversion of lactic acid into glucose, was proved to occur in the diabetic organism by Mandel and Lusk⁴. We may therefore represent the change as follows



It is generally conceded that the change represented above cannot

¹ We shall present the experimental details in a paper to be published immediately

² Embden and Kraus *Biochem Zeitschr*, xlv, p 1, 1912

³ This *Journal*, xi, p 361, 1912, and later papers

⁴ *Amer Journ of Physiol*, xvi, p 129, 1906

be direct but that intermediate products must take part in the reaction. Embden and his co-workers have considered the possibility of the formation of lactic acid from glyceric aldehyde and from dihydroxyacetone and have been able actually to demonstrate lactic acid production from these substances in the liver. On the other hand we have occupied ourselves with experiments concerning the possibility of lactic acid formation from methyl glyoxal and Levene and Meyer from their work on the action of leucocytes on sugars have been led to the same consideration. The possibility of methyl glyoxal being formed from glucose followed from Pinkus's observation of the formation of methyl glyoxal diphenylhydrazine on treating glucose with caustic soda in the presence of phenylhydrazine. The conditions of this experiment do not of course preclude complicated rearrangements, but recently we have been able to demonstrate the presence of methyl glyoxal in distillates obtained from the simple treatment of glucose with sodium phosphate solution. Moreover, Nef held the view that methyl glyoxal was the precursor of lactic acid in biochemical reactions,⁶ although he apparently abandoned this idea later.⁷

We⁸ have shown that small amounts of tissue extracts, acting under suitable conditions, may transform almost quantitatively many grams of methyl glyoxal into lactic acid in the course of a few minutes, so that no question of possible bacterial action need be considered. We have further shown that blood cells effect the same change, and that the active agent is an enzyme which is active in the intact animal. The enzymes we have named "Glyoxalase".

Since then we have been able to show the formation of abundance of lactic acid on perfusing a surviving liver with blood containing methyl glyoxal. It is a noteworthy fact that in every case we have obtained a mixture of *d* and *l* forms of lactic acid, and, as in our previous paper, we are inclined to believe in the presence of more than one enzyme of the type of glyoxalase.⁹ Later in

⁵ *Ber d deutsch chem Gesellsch*, **xxxi**, p 31, 1893

⁶ *Liebig's Annalen*, **cccxxxv**, p 279, 1904.

⁷ *Ibid*, **ccclvii**, p 305, 1907

⁸ *This Journal*, **xiv**, p 155, 1913

⁹ It is noteworthy that Embden, Baldes and Schmitz obtained mixtures of *l* and *d* lactic acid from glyceric aldehyde. *Biochem Zeitschr*, **xiv**, p 108, 1912

this paper evidence will be adduced showing that many glyoxals other than methyl glyoxal may be derived from amino-acids so that it is probable that separate catalysts of the type of glyoxalase may be concerned in their metabolism. It should be noted however that in continuation of their previous work Levene and Meyer have succeeded in showing that leucocytes and kidney tissue may convert methyl glyoxal into lactic acid containing an excess of the dextro component, *i e*, the lactic acid commonly encountered in animal tissues¹⁰

Finally we have been able to show that methyl glyoxal and also *l* lactic acid when given to the diabetic animal yield glucose, thus furnishing another link in the chain of reversible reactions. Whether methyl glyoxal is converted into glyceric aldehyde prior to glucose synthesis is at present undecided but appears probable.

There still remained the question, can lactic acid yield methyl glyoxal? While we can hardly hope at present to detect methyl glyoxal in the animal body we are able as the result of new experiments *in vitro* to show that this reaction is readily brought about. In other words, *the conversion of methyl glyoxal into lactic acid is a reversible reaction*¹¹. It is likely therefore that the action of glyoxalase is also reversible. We were able to demonstrate the conversion of lactic acid into methyl glyoxal by simple digestion of aqueous lactic acid at 37°C with nitrophenylhydrazine, a substance which forms an extremely insoluble derivative with methyl glyoxal¹².

We may therefore picture the relationships between glucose, methyl glyoxal and lactic acid as in the following scheme



But other substances than lactic acid have close metabolic relationships with glucose. Experiments upon glycosuric animals

¹⁰ This *Journal*, p 551, 1913. Since the appearance of our first communication on glyoxalase, an undated paper by Neuberg has appeared (*Biochem Zeitschr*, xlix, p 502, 1913) in which the conversion of methyl glyoxal into lactic acid is described. Apparently Neuberg was unaware of our earlier work.

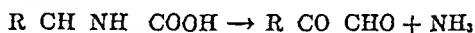
¹¹ Cf *Proc Chem Soc*, 1913, No 130.

¹² In addition to methyl glyoxal-dinitrophenylhydrazone, α -nitrophenylhydrazino-propionic acid and the nitrophenylhydrazone of pyruvic acid are formed. Their separation however is easy.

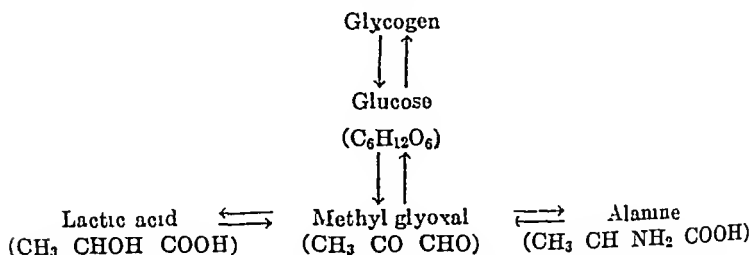
558 Metabolism of Carbohydrates and Proteins

especially by Lusk and Ringer, and later by one of us¹³ have shown that many amino-acids are apparently readily converted into glucose. In a recent paper it was suggested that methyl glyoxal might be an intermediate product in some cases at least. We have now obtained very definite evidence concerning a possible mode of conversion of an amino-acid such as alanine into sugar.

Every α -amino-acid we have thus far examined¹⁴ has furnished evidence that it may undergo dissociation to a limited extent at low temperatures in feebly acid solution with formation of α -ketonic aldehydes and ammonia



The demonstration of this reaction was only rendered possible by the use of a substance such as nitrophenylhydrazine which would remove the ketonic aldehyde from the reacting medium as quickly as it was formed. It is an extremely easy matter to prepare methyl glyoxal-dinitrophenylhydrazone from alanine on digesting it at low temperatures with nitrophenylhydrazine, preferably in the presence of a little acetic acid. There is little doubt that the reaction is reversible, so that we are now in a position for the first time to construct an approximate though doubtless incomplete scheme for the interconversion of alanine, lactic acid, methyl glyoxal and glucose by a series of reactions only involving the addition or removal of water or ammonia.

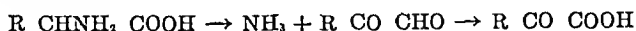


¹³ This Journal, *iv*, p. 321, 1913, *Zeitschr. f. physiol. Chem.*, *lxvi*, p. 106, 1910.

¹⁴ Including glycine, alanine, valine, leucine, aspartic acid and phenylalanine. The reaction in the case of glutamic acid is complicated by the fact that, as expected, one of its two carboxyl groups is retained and the product is relatively soluble.

The above scheme is of interest in connection with the mechanisms for the maintenance of approximate neutrality in living cells. It should be noted that alanine may be converted into "neutral" methylglyoxal with liberation of alkaline ammonia, while the methylglyoxal in turn may be converted into acid lactic acid by union with water. Furthermore we have found by experiments *in vitro* that the production of lactic acid from methyl glyoxal is promptly checked unless provision is made for the neutralization of the free acid. In addition we find that the conversion of alanine into methyl glyoxal and ammonia is greatly accelerated by acids. Thus it is clear that we are dealing with an exquisitely delicately adjusted condition of equilibrium provided with automatic checks guarding against undue development of either acid or alkali.

The demonstration of the conversion of amino-acids into α -ketonic aldehydes seems to us to be of importance in many connections. *It appears probable that α -ketonic aldehydes may represent the first step in the metabolism of amino-acids.* This hypothesis furnishes a reasonable explanation of the mechanism of the conversion of α -amino-acids into α -ketonic acids, a reaction so clearly demonstrated to occur in the animal organism by Neubauer,¹⁵ and later by Knoop. It is only necessary to assume the formation of the corresponding α -ketonic aldehyde from the amino-acid and oxidation of the former. Thus we have observed the formation of phenyl glyoxylic acid on perfusing a liver with blood containing phenyl glyoxal.



Furthermore it shows why the corresponding hydroxy-acids are not obligate steps in the catabolism of amino-acids, for it is known, for example, that α -hydroxy-phenylpropionic acid is relatively resistant to change in the body, while phenylalanine and phenylpyruvic acid are readily oxidized.

In addition it indicates a probable mode of synthesis of amino-acids in living cells from nitrogen-free ketonic aldehydes and ammonia.¹⁶ Thus it is known that lactic acid and alanine are inter-

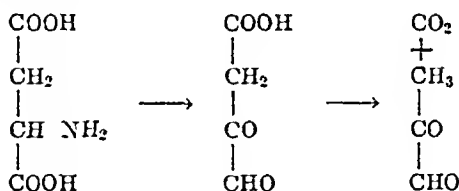
¹⁵ *Deutsch Arch f klin Med*, xciv, p 211, 1909, *Zeitschr f physiol Chem*, lxxvii, p 489, 1910, lxxi, p 153, 1911

¹⁶ We are at present engaged on investigations of this character

convertible in the animal body¹⁷ The scheme on p 558 clearly indicates a probable mechanism for the reaction

The formation of α -ketonic aldehydes from amino-acids has an interesting connection with the synthesis of pyrimidine derivatives in the body, as was pointed out in the case of methyl glyoxal derivable from glucose by Knoop and Windaus

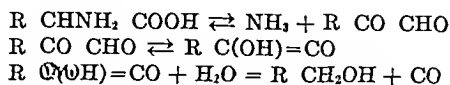
Again, the formation of glucose from various amino-acids in the diabetic organism is rendered much more intelligible The conversion of alanine into glucose has already been referred to The case of aspartic acid is particularly instructive Lusk and Ringer have shown that three out of the four carbon atoms of aspartic acid are convertible into glucose, and this is precisely what would be expected on the basis of β -ketonic aldehyde acid formation, for the substance formed, being a β -ketonic acid, would undoubtedly lose carbon dioxide, yielding methyl glyoxal We have had no difficulty in actually demonstrating the conversion of aspartic acid into a glyoxal-dinitrophenylhydrazone *in vitro* Aspartic acid is therefore at once brought into biochemical relation with glucose, lactic acid and alanine



Finally the relation of α -ketonic aldehydes to alcohol formation from amino-acids and from sugar may be mentioned Buchner and Meisenheimer's original experiments on the fermentation of methyl glyoxal are unconvincing in the light of present knowledge, since we have shown that much acid may be formed from glyoxals by the action of glyoxalase present in the yeast cells New experiments are undoubtedly necessary In the meantime it would seem a more attractive hypothesis to assume that the conversion of amino-acids into alcohols as observed by Felix Ehrlich proceeds

¹⁷ Neuberg and Langstein *Arch f (Anat u) Physiol*, Suppl Band, 1903, p 514, Embden and Kraus *loc cit*

by way of the ketonic aldehydes rather than by the hydroxy-acids as has been assumed



We are at present engaged in testing this hypothesis experimentally

SUMMARY

1 It is shown that by a suitable choice of experimental conditions it is possible to convert at low temperatures α -amino- and α -hydroxy-acids into α -ketonic aldehydes. Lactic acid and alanine for example, yield methyl glyoxal.

2 Methyl glyoxal is acted upon by enzymes named "glyoxalases," present in the animal body, with formation of both *d*- and *l*-lactic acid. When given to the glycosuric animal methyl glyoxal and both *d* and *l* lactic acids yield glucose. Methyl glyoxal is believed, therefore, to be an intermediate product in the mutual interconversion of alanine, lactic acid and glucose.

The relationship in the living cell between alanine, lactic acid, methyl glyoxal and glucose is believed to constitute a delicately adjusted equilibrium concerned with the maintenance of approximate neutrality. The view is advanced that α -ketonic aldehydes may represent the first step in the metabolism of amino-acids, and that the formation of these substances furnishes an adequate explanation of the origin of α -ketonic acids from α -amino-acids. Furthermore it affords an explanation of the fact that α -hydroxy-acids are not obligate steps in the catabolism of most α -amino-acids.

The relation of α -ketonic aldehyde formation is discussed in connection with the mechanism of glucose production in the glycosuric animal, with the synthesis of pyrimidine derivatives, and in connection with the mechanism of alcoholic fermentation.

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